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Estimation of the phospholipid distribution in the human platelet plasma membrane based on the effect of phospholipase A_2 from *Naja nigricollis*

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Human platelets in three physiological states were prepared. These states were the gel-filtered, the thrombin-induced shape-changed, and the thrombin-activated platelets. The phospholipid distributions in these three types of membrane were probed by using the basic phospholipase A_2 of *Naja nigricollis*. This enzyme could penetrate through these membranes to hydrolyze all of their accessible phospholipids and to cause cell lysis. The hydrolytic time-courses displayed three phases. The state of platelet in each lipid hydrolytic phase was examined by: (1) measuring the leakage of lactate dehydrogenase; (2) analyzing the morphology by both scanning and transmission electron microscopy (scanning EM and transmission EM); and (3) estimating the hydrolysis of the [^{32}P]phosphate-labeled platelets. The existence of these three hydrolytic phases may signify that the phospholipase A_2 sequentially hydrolyzed its substrates in the membrane outer leaflet, in the inner one, and in the cytosol. The content and the distribution of each phospholipid class in the plasma membranes of the resting and of the shape-changed platelets were similar. These membrane surfaces consisted mainly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Phosphatidylserine (PS) was not exposed on the surface of the shape-changed platelet. The content of each lipid class in the activated platelet membrane was 10% more than that in the resting platelet. PS was found on the activated platelet cell surface. This implies that PS is exposed only during platelet secretion.

Introduction

Membrane components are asymmetrically distributed in the lipid bilayer [1,2]. The distributions of phospholipids in the resting human platelet plasma membrane have been documented [3–8]. Platelets, when activated by agonists, undergo a change in shape and secrete the contents of the intracellular granules [9]. An increase in the lipid content of the activated platelet membrane has been reported [8]. The exposure of phosphatidylserine (PS) in the cell surface of the activated platelets is suggested to result from a flip-flop mechanism [8]. Whether PS is exposed on the membrane surface of the shape-changed platelets was investigated in this study. Platelets in three different physiological states were prepared. They were the resting gel-filtered, the thrombin-

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; EM, electron microscopy; SCOCS, surface-connected open canalicular system.

activated, and the irreversible shape-changed platelets. The last type was prepared by hirudin-thrombin stimulation [10]. The phospholipid distributions in these membranes were probed by the basic phospholipase A_2 ($pI = 10.6$) of *Naja nigricollis* [11].

The basic phospholipase A_2 is an enzyme known to be membrane-lytic to erythrocytes as well as to other tissues [12]. This study showed that the phospholipase A_2 could penetrate through the platelet membranes as well. The enzyme completely hydrolyzed the platelet phospholipids, displaying triphasic time-course profiles, which may well signify the enzyme's sequentially hydrolyzing its substrates in the different cell membrane compartments. Hence, the phospholipid distributions in three types of platelet plasma membrane were estimated.

Experimental procedures

Materials

Chemicals and organic solvents were purchased from the E. Merck (Darmstadt, F.R.G.). All organic solvents were redistilled before use. The following reagents were obtained from the Sigma (St. Louis, MO, U.S.A.): bovine thrombin, collagen (type III from calf skin), hirudin, NADH, lactate dehydrogenase (from porcine muscle type XXIX), fatty-acid-free bovine serum albumin. Sepharose 2B was from Pharmacia (Piscataway, NJ, U.S.A.). The basic phospholipase A_2 was purified from the snake venom of *Naja nigricollis* as described previously [11]. 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, China.

Methods

Preparation of platelets in three physiological stages. 9 vol. of the 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 $250 \times g$ for 15 min, at room temperature. The resting platelets were isolated from platelet-rich plasma by gel-filtration according to the method of Large et al. [13]. Unless otherwise specified, the buffer solution used in this study was modified calcium-free Tyrode's buffer containing 0.1% dextrose and 0.2% of bovine serum albumin [13]. 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 carrier-free [^{32}P]phosphate for 1 h prior to the gel-filtration. The cell number was estimated in a hemacytometer by a phase-contrast microscope (Type 104, Nikon, Tokyo, Japan).

The thrombin-activated platelets were prepared as described previously [14]. In brief, the gel-filtered platelets ($1.5 \cdot 10^8$ cells/ml) were suspended in 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 incubation the suspension was centrifuged. The pellet was resuspended in buffer containing 2 mM EDTA (pH 7.4).

The shape-changed platelets were prepared by a modification of the study of Holmson et al. [10]. 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. After another minute, thrombin (0.05 U/ml) was introduced. The suspension was then centrifuged. The pellet was resuspended in the buffer.

Hydrolysis of platelet phospholipids by phospholipase 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 the plasma. Each physiological type of platelet suspension ($1.5 \cdot 10^8$ cells/ml) 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 the phospholipase A_2. The reaction was stopped by introducing 2 mM of 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, followed immediately by 3 mM Ca^{2+} to initiate the reaction. The reaction was terminated by adding 2 mM of EDTA, and chilling in ice. The time-course of the hydrolysis of each lipid class was followed. A control experiment was performed by incubating the platelets with the enzyme alone, without adding Ca^{2+}. The experiment showed that the phospholipase A_2 could not hydrolyze lipid, in the absence of Ca^{2+}, in a span of 3 h incubation.

Analysis of lipid classes. Lipids were extracted

from the hydrolytic reaction medium according to the method of Bligh and Dyer [15]. Each lipid class was identified by the two-dimensional thin-layer chromatography. The thin-layer plate was prepared by the impregnation of silica-gel 60 H with 0.9% of magnesium acetate. The solvent systems were chloroform/methanol/25% ammonia/H_2O (60 : 35 : 4 : 1, v/v) in the first dimension and chloroform/methanol/glacial acetic acid (60 : 27 : 8, v/v) in the second. The chromatograms were visualized by iodine.

The quantification of phospholipid was accomplished by the estimation of phosphorus content as described previously [16,17]. Each hydrolyzed phospholipid class was expressed as the percentage of its total amount in the platelet. The estimations of hydrolyzed PS and PI were 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 lipid phosphorus from the human erythrocyte lipid were 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.).

Other analyses. The effect of the phospholipase on the physiological functions of the resting platelet was studied by using the Aggrecorder (Model PA-3210, Hitachi, Kyoto, Japan). The estimation of the lactate dehydrogenase activity in the supernatant of the reaction medium was made according to the method described by Bergmeyer et al. [18]. EDTA did not affect the lactate dehydrogenase activity. The control systems were performed by incubating the platelets either with phospholipase A_2 alone, or with 1 mM Ca^{2+} alone. In the determination of the lactate dehydrogenase activity, the enzymatic activity in the supernatant of the 0.1% Triton X-100-treated platelets was used as 100% of the dehydrogenase activity. No significant leakage (less than 3%) of the dehydrogenase was found in the control experiments in 3 h incubation.

Morphological studies by electron microscopy. The sample preparations were modified from that described by White et al. [19]. In brief, the phospholipase A_2-treated platelets were added with 5 vol. of the ice-cold 2.5% glutaraldehyde in modified calcium-free Tyrode's buffer (pH 7.4), and fixed at 4°C for 30 min. The suspension was then centrifuged at $1000 \times g$ for 20 min at 4°C. The pellets were post-fixed with 1% of osmium tetroxide in the modified buffer (pH 7.4) for 1 h, and then were dehydrated. The samples were embedded in the Spurr's resin [20]. Sections were cut on LKB 2088 Ultramicrotone using a glass knife. The sections were stained with 5% of methanolic uranyl acetate followed by Reynolds lead citrate [21]. The stained samples were examined in a Hitachi H-600 transmission electron microscope, at 75 kV. For the scanning EM study, the samples were dried in a critical-point dryer (Hitachi HCP-2, Japan) in CO_2, and then plated with gold in a Ion Coater (Eiko Engineering, model IB-2). The plated samples were studied under a Hitachi S-520 scanning electron microscope at 20 kV.

Results

Effect of phospholipase A_2 on the gel-filtered platelets

Basic phospholipase A_2, in the presence of 1 mM Ca^{2+} and 0.02% Triton X-100, hydrolyzed more than 90% of each phospholipid class in the gel-filtered platelets (Fig. 1). This indicated that the enzyme possessed no substrate specificity toward these phospholipids. In the absence of detergent, the enzyme hydrolyzed its substrates in three phases (Fig. 1). In the first phase, the enzyme hydrolyzed mainly PC, PE, and PI, 38%, 30% and 20%, respectively. No significant amount (3%) of PS was hydrolyzed. A lag period of 30 min was followed. In the second phase, the enzyme hydrolyzed 23% more PC, 26% PE, 37% PI, and 50% PS. Another lag period followed before the phospholipase A_2 could hydrolyze all of the remaining phospholipids.

In each lipid hydrolytic phase, the state of platelet was analyzed by several experiments. Firstly, the lactate dehydrogenase activity in the supernatant of the hydrolytic medium was analyzed. In the first two hydrolytic phases, phospholipase A_2 did not cause any significant leakage of the cytoplasmic marker enzyme (Fig. 2). How-

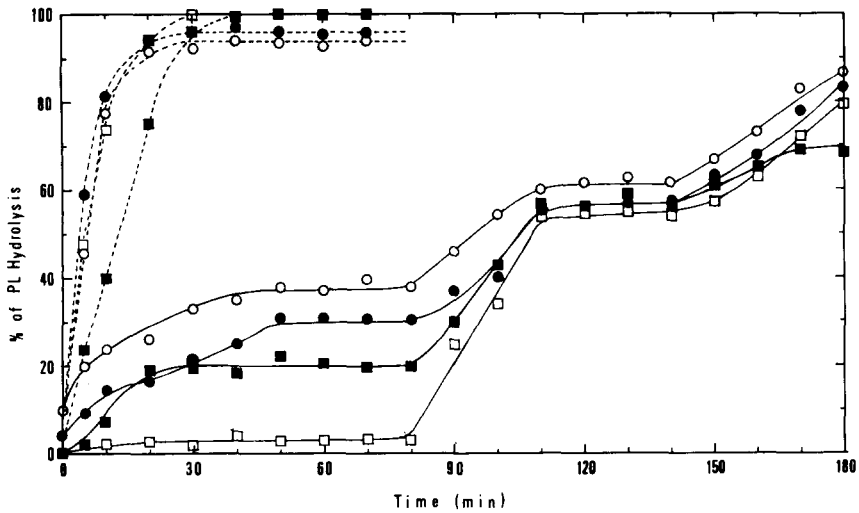


Fig. 1. The lipid hydrolysis of gel-filtered platelets by phospholipase A_2. Details of the experiments are given in Experimental procedures. The phospholipid classes are PC (○), PE (●), PS (□) and PI (■). The dashed lines indicate the lipid hydrolysis found in the presence of 0.02% of Triton X-100.

ever, in the lag period of the second phase, 8% of the dehydrogenase activity was detected in the supernatant. In the third phase, more than 20% of the marker enzyme was found. Secondly, the morphological studies by both scanning and transmission EM (Fig. 3) showed that the results were

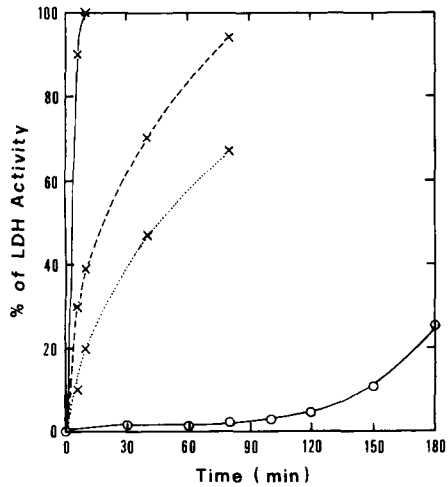


Fig. 2. The time-course of lactate dehydrogenase activity appearing in the supernatants of both phospholipase A_2- (○) and Triton X-100- (×) treated resting platelets. The enzymatic activity found in the supernatant of the 0.1% Triton X-100-treated platelets was used as 100%. Details of the experiments are given in Experimental procedures. The various concentrations of detergent used were: 0.02% (× ——— ×), 0.014 (× — — ×) and 0.01 (× · · · · · ×).

comparable to those of the estimation of the cytoplasmic marker enzyme. In the first hydrolytic phase, phospholipase A_2 induced the resting platelet into a puffy disc with pseudopods (Fig. 3C), and the inside of the cell was intact (Fig. 3D). In the second phase, the enzyme caused the cell to become irregular in shape, with more pseudopods, and with dilated holes of the surface-connected open canalicular system (SCOCS) (Fig. 3E). For the rest, the organelles were still evently dispersed inside the cell (Fig. 3F). The lysed cells were found to be less than 5%. In the third phase, the enzyme caused more than 45% of the cells to lyse (Fig. 3G and H). Thirdly, the aggregation studies showed that phospholipase A_2 immediately induced irreversible shape change in resting platelets (Fig. 4). In the second hydrolytic phase, phospholipase A_2 could induce the resting platelets to aggregation. Indomethacin could prevent the platelets from this phospholipase A_2-induced aggregating. The enzyme-treated platelets, after washing, were still sensitive to the stimulation of agonists such as thrombin and collagen (Fig. 5B–F). These results imply that the plasma membrane was well preserved after the second phase of lipid hydrolysis.

Finally, the [^{32}P]phosphate-labeled platelets were used to investigate the physical aspects of lipid hydrolysis by phospholipase A_2 (Fig. 6). In the first hydrolytic phase, phospholipase A_2

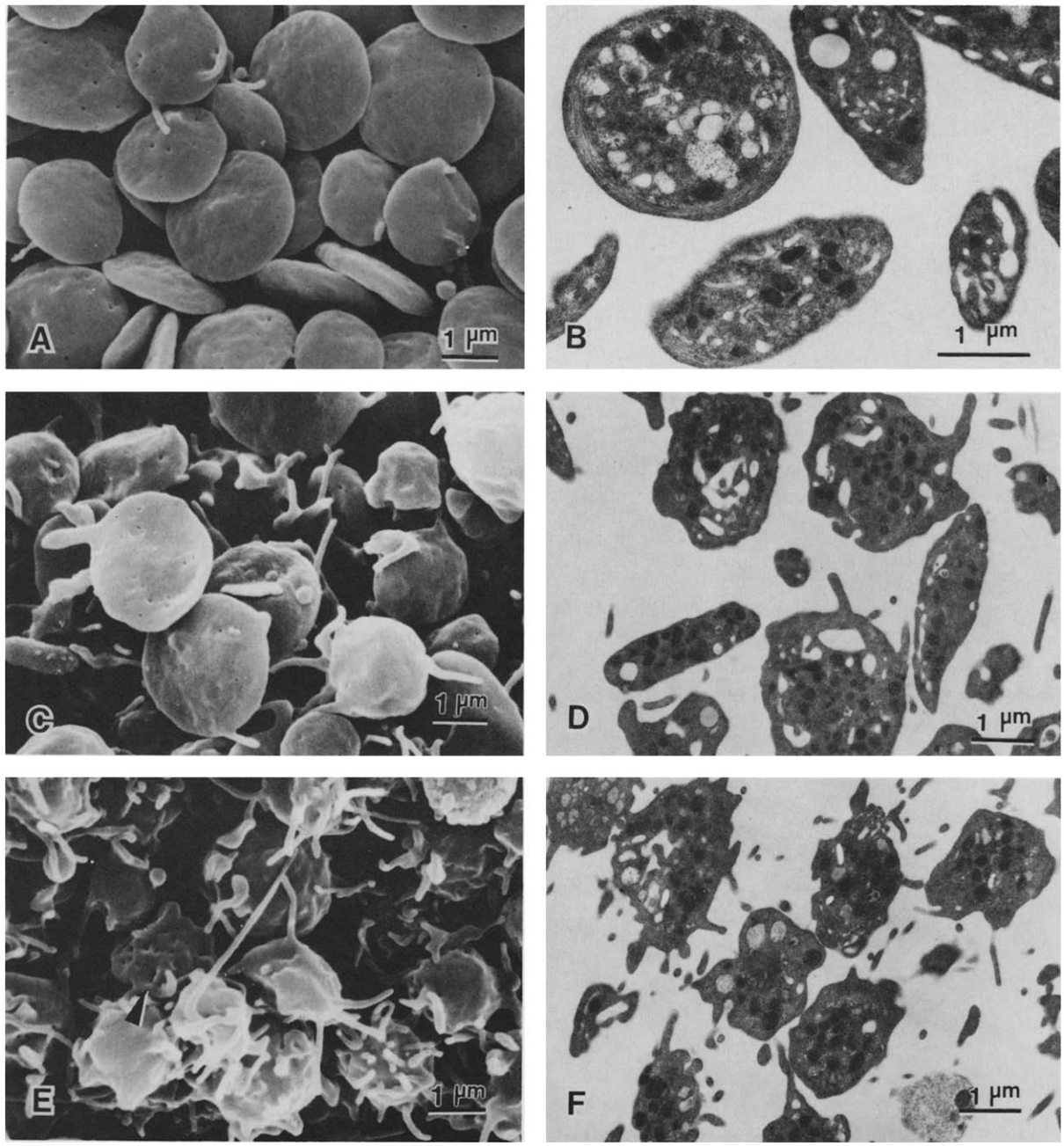
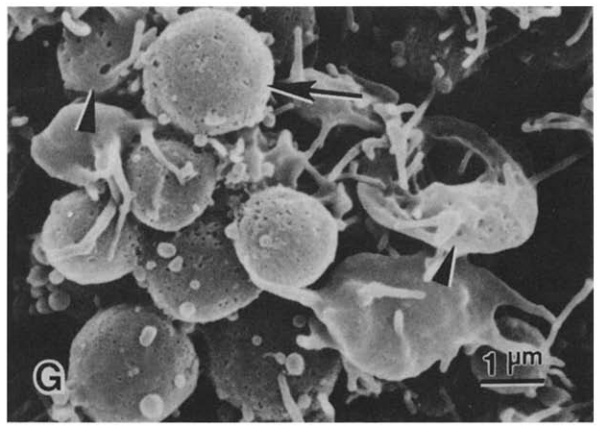
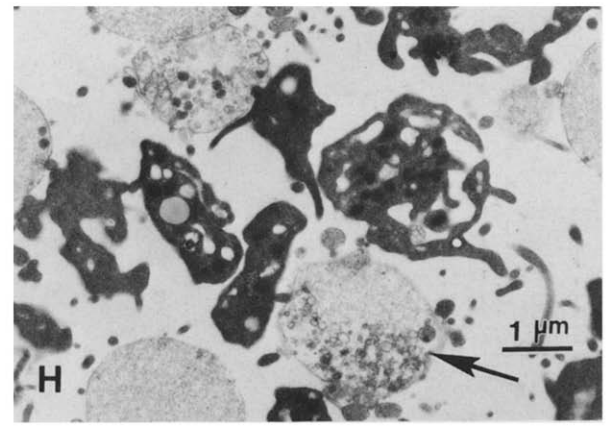


Fig. 3. Electron micrographs of the phospholipase A_2-treated resting platelets studied by scanning EM ($\times 9000$ for A, C, E, G) and by transmission EM ($\times 15000$ for B; and $\times 10000$ for D, F, H). Details of the experiments are given in Experimental procedures. In the presence of 10 μM indomethacin, the resting platelets (A and B) were treated with phospholipase A_2 for 50 min (C and D), 120 min (E and F), and 180 min (G and H). Arrows indicate the lysed cells, while the arrow-heads indicate the cells with dilated SCOCS (surface-connected open canalicular system) holes on the cell surface.





enhanced the ^{32}P-incorporation rates of all the phospholipid classes in platelets. The enzyme started to inhibit the incorporation of [^{32}P]phosphate into phospholipids in the second phase. It completely damaged the metabolic machinery of the phospholipid when it started its third hydrolytic phase. For example, phospholipase A_2 in its first hydrolytic phase accelerated the ^{32}P-incorporation rates in both PI and PA (Fig. 6A). The enzyme started to hydrolyze [^{32}P]PI in its second hydrolytic phase, while it inhibited only 80% of the incorporation rate in [^{32}P]PA. When phospholipase A_2 started to hydrolyze its third-phase substrate, the radioactivity in [^{32}P]PA was decreasing. Another example of the hydrolysis of [^{32}P]PC is

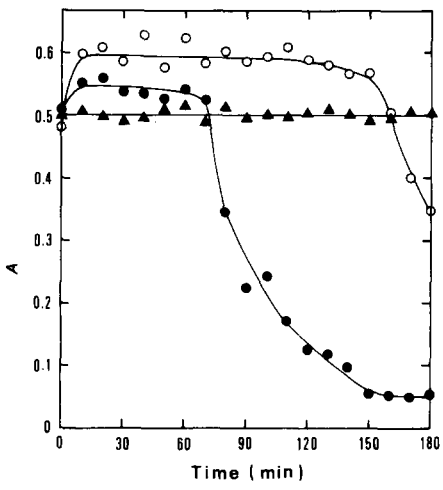


Fig. 4. The effect of phospholipase A_2 on gel-filtered platelets, estimated by the absorbance (A) of the cell suspension, recorded by the Aggrecorder. The initial absorbance of the cell suspension ($1.5 \cdot 10^8$ cells/0.5 ml) with 1 mM of Ca^{2+} (▲), was 0.50. The enzyme (1 µg) was added 2 min after the recording was started. The cell suspension was either in the presence (○), or in the absence (●), of 20 µM indomethacin.

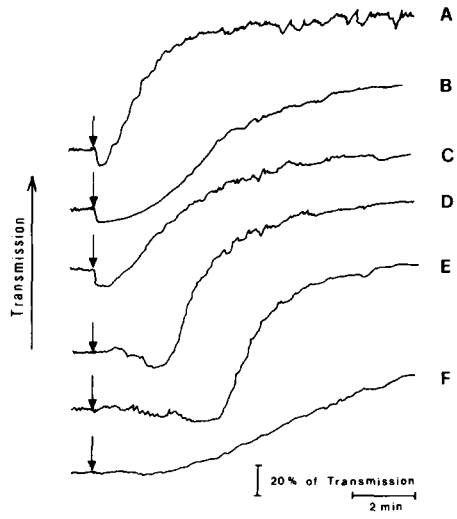


Fig. 5. The aggregation of phospholipase A_2-treated gel-filtered platelets. The cell suspension contained $1.5 \cdot 10^8$ cells in 0.5 ml of the modified Ca^{2+}-free Tyrodes buffer (pH 7.4). The arrows indicate when the agonists were added. In the control experiments (A and D), the gel-filtered platelets, after incubation at 37°C for 1 h, were stimulated with either 0.05 U thrombin (A) or 50 µg collagen (D), B and E show the resting platelets, which had been incubated with phospholipase A_2 for 1 h, and then stimulated with either 0.05 U thrombin (B) or 50 µg collagen (E). Figs. C and F show the resting platelets, which had been treated with phospholipase A_2 in the presence of 20 µM indomethacin for 2 h and washed, and then stimulated with either 0.05 U thrombin (C) or 50 µg collagen (F). The tracings were reproduced from those recorded by the Aggrecorder.

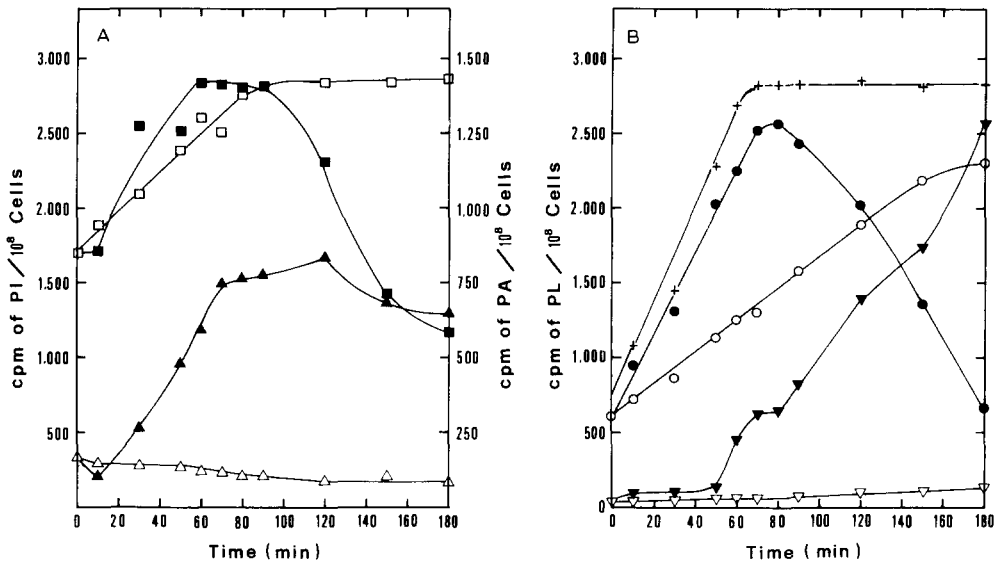


Fig. 6. The hydrolysis of ^{32}P-labeled gel-filtered platelets by phospholipase A_2. The solid symbols indicate the experiments in which the phospholipase A_2 was added. The open symbols represent the control experiments. Details of the experiments are given in Experimental procedures. (A) The hydrolysis of both PI (□, ■) and PA (△, ▲). (B) The hydrolysis of PC (○, ●) and lysoPC (▽, ▼). (+) indicates the sum of [^{32}P]PC and [^{32}P]lysoPC in those experiments in which phospholipase A_2 was present.

shown in the Fig. 6B. The incorporation rate of [^{32}P]phosphate into PC was also enhanced in the first hydrolytic phase. An increase in ^{32}P-labeled lysoPC was observed after a prolonged incubation, of 50 min. By that time, the radioactivity in PC was still increasing with time, but phospholipase A_2 had hydrolyzed almost all of its first-phase PC (cf. Fig. 1). The radioactivity in [^{32}P]PC began to decrease after 80 min of incubation. The decrease in the radioactivity in PC corresponded to the increase of that in lysoPC. This indicates that phospholipase A_2 completely inhibited the PC metabolic machinery in the second phase. These results indicated that the platelets in each lipid

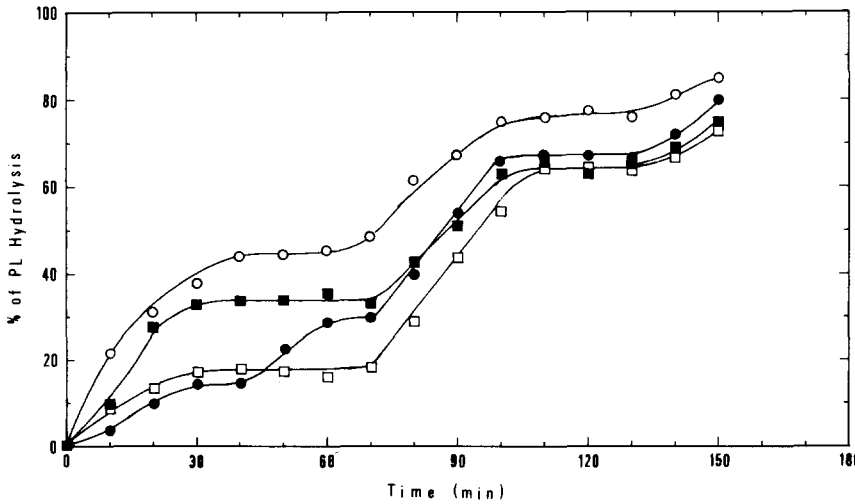


Fig. 7. Lipid hydrolysis of activated platelets by phospholipase A_2. Details of the experiments were described in Experimental procedures. The phospholipid classes are PC (○), PE (●), PS (□), and PI (■).

hydrolytic phase exhibited different properties. The triphasic hydrolysis may well mean that the enzyme sequentially hydrolyzed its substrates in the different compartments of the resting platelet.

The effect of phospholipase A_2 on thrombin-activated platelets

Phospholipase A_2 also hydrolyzed lipids in the activated platelets with triphasic time-course profiles (Fig. 7). In the first two phases, however, the enzyme hydrolyzed 10% more of each lipid class in the activated platelets than in the case of the resting platelets. Moreover, the enzyme hydrolyzed 18% of the PS in the hydrolytic phase.

In each phase, the enzyme caused the activated platelets to display different lipid metabolic activities. In the first phase, the enzyme enhanced the ^{32}P-incorporation rate in both PI and PA (Fig. 8A). It did not enhance the ^{32}P-incorporation rate in PC (Fig. 8B), nor in the other phospholipids. In the second phase, the enzyme began to inhibit the incorporation rate in both PI and PC. The enzyme damaged the activated-platelet metabolic machinery in the third phase.

The activated platelets also exhibited different morphology in each hydrolytic phase (Fig. 9). In the first hydrolytic phase, the phospholipase gave rise to more pseudopods on the cell surface (Fig. 9C). The cell internal was intact (Fig. 9D). In the lag period of the second phase, the enzyme caused more than 30% of the activated platelet to lyse (Fig. 9E and 9F). No significant leakage of the lactate dehydrogenase was detected in the first two phases. When the cells were treated with the enzyme for 130 min, 15% of the cytoplasmic marker enzyme was found to have leaked out.

The results also suggested that the phospholipase A_2 might sequentially hydrolyze its substrates in the different compartments of the activated platelets.

The shape-changed platelets affected by the phospholipase A_2

The phospholipase A_2 hydrolyzed its substrates in the shape-changed platelets with triphasic time-course profiles, also (Fig. 10). The lipid hydrolysis patterns were similar to those found for the hydrolysis of the lipids in the resting platelets. No significant amount of PS was hydrolyzed in the first phase.

Using the ^{32}P-labeled platelets as substrate, the effect of enzyme on the shape-changed platelets

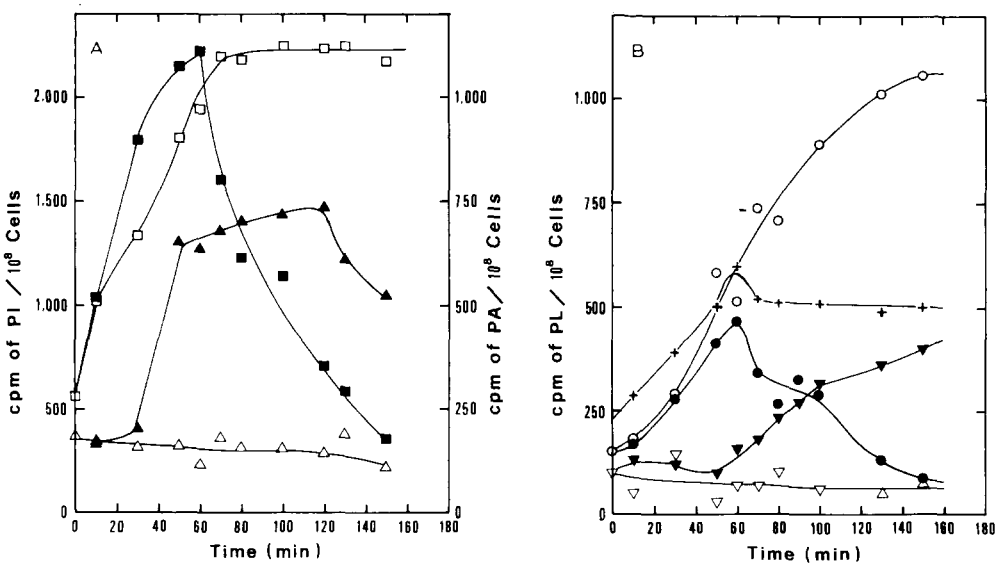


Fig. 8. The hydrolysis of [^{32}P]phosphate-labeled activated platelets by phospholipase A_2. The solid symbols indicate those experiments in which the phospholipase A_2 was added. The open symbols represent the control experiments. Details of the experiments are given in Experimental procedures. (A) The hydrolysis of both PI (□, ■) and PA (△, ▲). (B) The hydrolysis of PC (○, ●) and lysoPC (▽, ▼). + indicates the sum of [^{32}P]PC [^{32}P]lysoPC found in the experiments in which phospholipase A_2 was present.

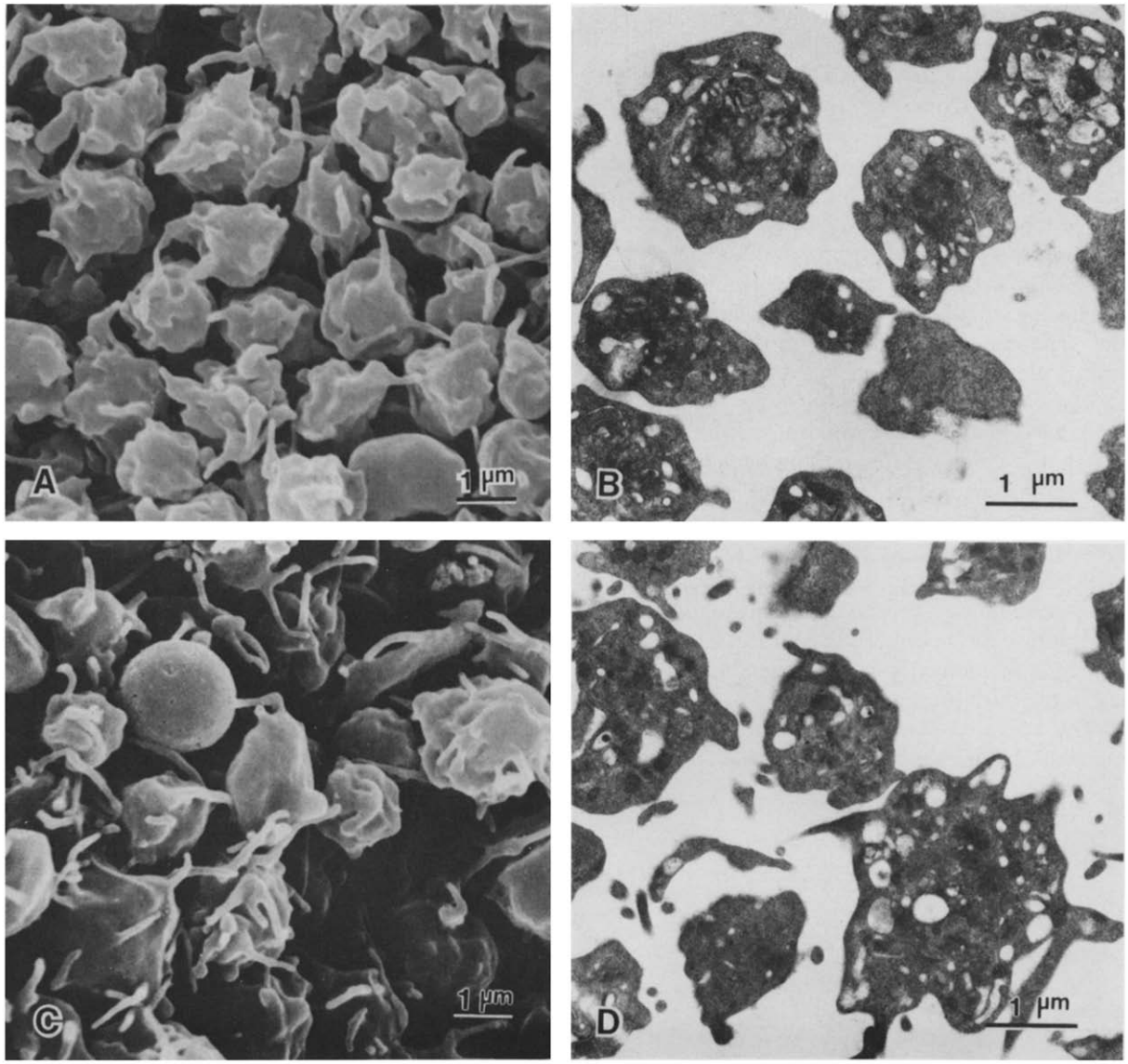
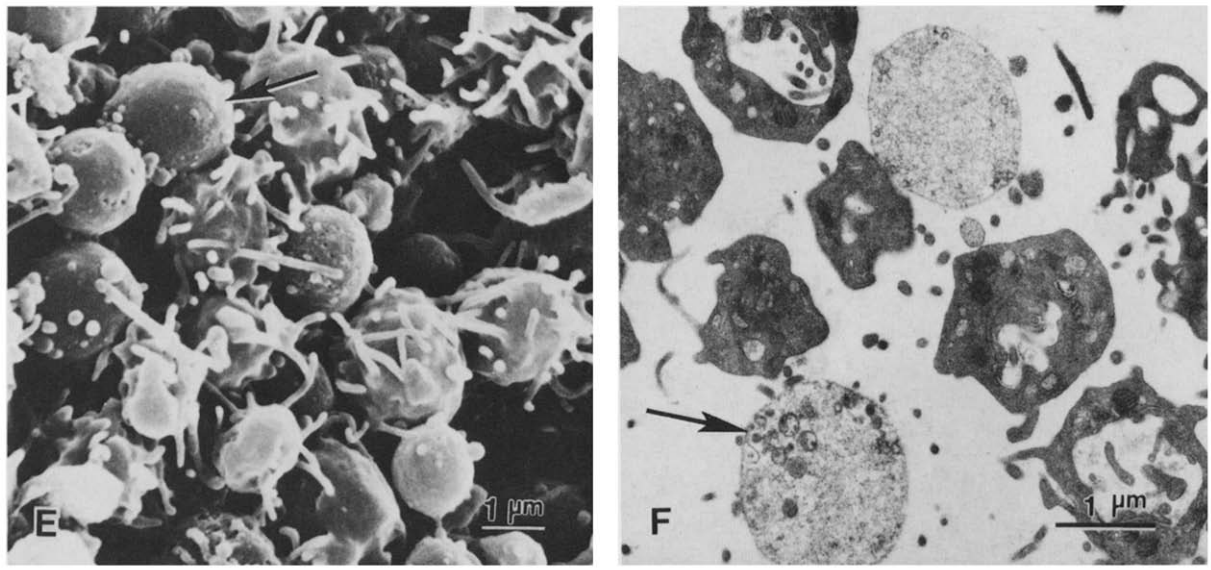


Fig. 9. Electron micrographs of phospholipase A_2-treated activated platelets studied by both scanning EM (×9000 for A, C, E) and transmission EM (×14000 for B, D, F). Details of the experiments are given in Experimental procedures. The activated platelets (A and B), in the presence of 10 μM indomethacin, were treated with the enzyme for 60 min (C and D), and 130 min (E and F). Arrows indicate the lysed cells.

was similar to that on the resting platelets (Fig. 11). In the first phase, it enhanced the ^{32}P-incorporation rate of all phospholipids. The enzyme inhibited the metabolic machineries in the second phase. It damaged the metabolic machinery in the third one (Fig. 11). The increase in the radioactivity in lysoPC was observed only after the enzyme had almost completely hydrolyzed its first phase substrates (compare Figs. 10 and 11B).

Morphological studies showed that the shape-changed platelets were deformed, to become either puffy discoid or irregular in shape (Fig. 12A). Transmission EM revealed that the dispersed organelles were darker than those in the resting platelets (Fig. 12B). Phospholipase A_2, in its first hydrolytic phase, did not induce any significant



increase of pseudopods, but the SCOCS holes began dilate in about 10% of the cells (Fig. 12C). The organelles were evenly distributed in the cytosol (Fig. 12D). In the second phase, the enzyme induced the platelets with more pseudopods and more dilated holes (Fig. 12E). In the lag period of the second phase, phospholipase A_2 caused 15% of the cells to lyse (Fig. 12E and F). No significant leakage of the lactate dehydrogenase was observed in the first two phases, while about 8% cytoplasmic enzyme was detected in the lag period of the second phase.

These results also suggested that phospholipase A_2 hydrolyzed its substrates in sequence. They also indicate that PS was not exposed on the shape-changed platelet cell surface.

In summary, the triphasic lipid hydrolysis by basic phospholipase A_2 might indicate that the enzyme hydrolyzed the phospholipids in the plasma membrane outer leaflet in its first hydrolytic phase.

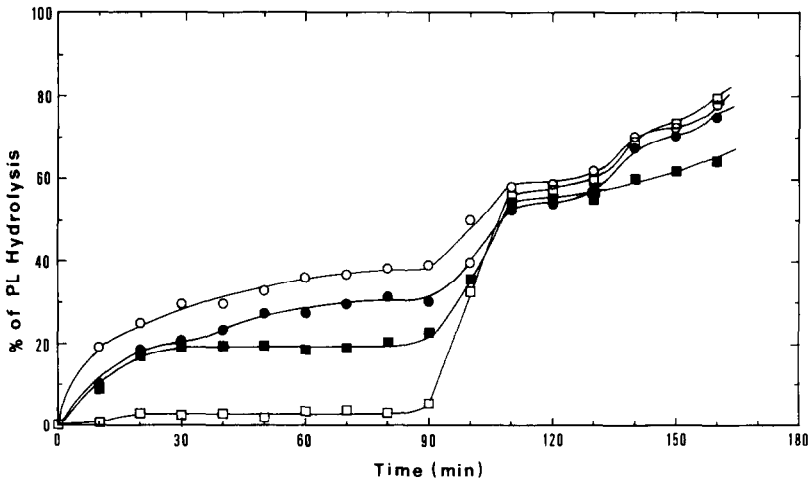


Fig. 10. Lipid hydrolysis of the shape-changed platelets by phospholipase A_2. Details of the experiments are given in Experimental procedures. The lipid classes are PC (○), PE (●), PS (□), and PI (■).

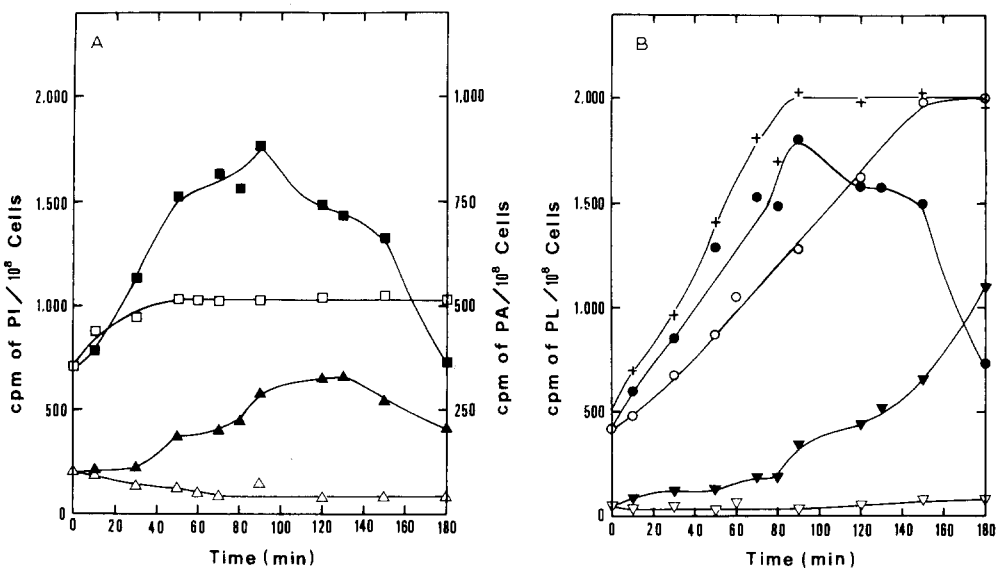


Fig. 11. The hydrolysis of [^{32}P]phosphate-labeled shape-changed platelets by phospholipase A_2. The solid symbols indicate those experiments in which enzyme was added. The open symbols represent the control experiments. Details of the experiments are given in Experimental procedures. (A) The hydrolysis of both PI (□, ■) and PA (△, ▲). (B) The hydrolysis of PC (○, ●) and lysoPC (▽, ▼). (+) indicates the sum of [^{32}P]PC and [^{32}P]lysoPC, found in those experiments in which enzyme was present.

TABLE I

THE ASYMMETRIC DISTRIBUTIONS OF PHOSPHOLIPIDS IN THE HUMAN PLATELET PLASMA MEMBRANE

Data (mean ± S.D.) are taken from at least ten independent experiments of the hydrolysis of each phospholipid (PL) class by the basic phospholipase A_2 of *N. nigricollis* (p*I* = 10.6). The three physiological types of platelet where the resting gel-filtered (GFP), the hirudin-thrombin-induced shape-changed (SCP), and the thrombin-activated (TAP) platelets. The percentage of each PL class in the plasma membrane was calculated from the plateau of the second hydrolytic phase, while that of each PL class in the outer leaflet was taken from the plateau of the first hydrolytic phase. Hence, the percentage of each PL class in the inner leaflet was obtained from the difference between plasma membrane and the outer leaflet.

Types of platelet	PL class	% of total of each PL class in platelet			Distribution (percentage) Outer/Inner
		Plasma membrane	Outer leaflet	Inner leaflet	
GFP	PC	61.0 ± 1.2	38.0 ± 1.2	23.0 ± 2.4	62.3/37.7
	PE	56.5 ± 1.5	30.5 ± 0.5	26.0 ± 2.0	54.0/46.0
	PS	54.0 ± 1.0	3.2 ± 0.8	50.8 ± 1.8	5.9/94.1
	PI	57.5 ± 2.0	20.0 ± 2.5	37.5 ± 4.5	34.7/65.3
SCP	PC	60.0 ± 1.5	38.0 ± 1.5	22.0 ± 3.0	63.3/36.7
	PE	55.5 ± 0.5	30.0 ± 1.5	25.5 ± 2.0	54.0/46.0
	PS	58.0 ± 2.0	3.0 ± 1.0	55.0 ± 3.0	5.2/94.8
	PI	55.0 ± 0.5	19.7 ± 1.3	35.3 ± 1.8	35.8/64.2
TAP	PC	71.2 ± 1.3	45.0 ± 0.8	26.2 ± 2.1	63.2/36.8
	PE	66.7 ± 0.8	30.2 ± 0.4	36.5 ± 1.2	45.3/54.7
	PS	64.0 ± 0.5	18.0 ± 0.5	46.0 ± 1.0	28.1/71.9
	PI	64.0 ± 0.5	34.5 ± 1.5	29.5 ± 2.0	53.9/46.1

It sheared off those in the inner leaflet in the second one. Finally, it hydrolyzed the phospholipids inside the cytosol in the third phase and caused cell lysis. Based on this assumption, the asymmetric distributions of each phospholipid class in three types of platelet plasma membrane are compiled in Table I.

Discussion

Platelets, when they are stimulated by agonists, change their shapes, and then secrete out the contents in the internal organelles. The three physiological types of human platelet, i.e., the resting, the shape-changed, and the activated platelets, were prepared. The phospholipase A_2 of *N. nigricollis* can penetrate through these membranes upon prolonged incubation. It causes: (1) hydrolysis of more than 90% of the platelet phospholipids (Figs. 1, 7, 10); (2) damage of the phospholipid metabolic machinery (Figs. 5, 8, 11); (3) cell lysis revealed by both scanning and transmission EM (Figs. 6, 9, 12); and (4) leakage of the lactate dehydrogenase from the cell (Fig. 2).

This study has shown that the basic phospholipase A_2 hydrolyzes its substrates with triphasic time-course profiles. A membrane-penetrating protein may have a dual conformation [22]. If the phospholipase A_2 has a partition rate lower than its enzymatic rate, the time-course of its hydrolyzation will be observed as two phases. The lag period is determined by the partition coefficient. Another lag period will also be shown for the enzyme to pass from its lipid environment into the aqueous one. Therefore, it is plausible for a membrane-penetrating phospholipase to display such triphasic hydrolytic patterns.

The triphasic lipid hydrolysis by basic phospholipase A_2 may mean that the enzyme sequentially hydrolyzed its substrates in the membrane outer leaflet, in the inner leaflet, and in the cytosol. In the first hydrolytic phase, the cells are not lysed (Figs. 2, 3, 9, 12), and the lipid metabolic machineries are not damaged (Figs. 6, 8, 11). The phospholipase-treated resting platelets retain their sensitivity to agonist stimulation (Figs. 3 and 5). We have also found that phospholipase A_2 does not affect the Ca^{2+}-uptake of resting platelets during incubation with cells for 80 min (data not shown). Hence, the substrates hydrolyzed in the first phase may represent the phospholipids in the plasma membrane outer leaflet. In the second phase, the enzyme does not cause any significant cell lysis, either. However, calcium ions are found to leak out of the resting platelets after 90 min incubation (data not shown). The enzyme also causes the cell to change its morphology as compared with that in the first phase. Furthermore, the platelets treated with phospholipase A_2 are still sensitive to the stimulation of agonists (Fig. 5). In this second hydrolytic phase, the enzyme may be in hydrophobic conformation. It may act as a plug in the lipid bilayer and retain hydrolytic activity similar to that of its hydrophilic form. Hence, it hydrolyzes the substrates in the membrane inner leaflet without causing the leakage of the lactate dehydrogenase. In the third phase, the enzyme partitions into the cytosol to cause cell lysis and to hydrolyze all the remaining substrates. Based on this hypothesis, each phospholipid class in the resting platelet plasma membrane is at about 55% of its total in platelets. The value obtained is in agreement to the findings by Perret et al. [6]. The content of each phospholipid class in the shape-changed platelet membrane is similar to that in the resting platelets. The content of each lipid class in the activated platelet membrane is about 10% more than that in the resting platelet membrane. It confirms the findings by Bever et al. [8].

Phospholipids in the plasma membranes of the resting and the shape-changed platelets are in a similar asymmetric distribution (Table I). The contents of both PC and PE are higher in the membrane outer leaflet than in the inner one. This is in discrepancy with the findings from previous studies [5,6,8]. This may reflect the increased ability of the phospholipase A_2 of *N. nigricollis* to perform extensive degradation of phospholipids in the outer leaflet. Another possibility is that the basic phospholipase A_2 may induce the transmembrane movement of some phospholipids from the inner leaflet. It has been suggested that the accumulation of free fatty acid in a membrane can promote the action of phospholipase C [23,24]. The activated endogenous phospholipase C will cause the accumulation of diacylglycerol, which is able to induce the flip-flop of PS [25]. This may explain the high

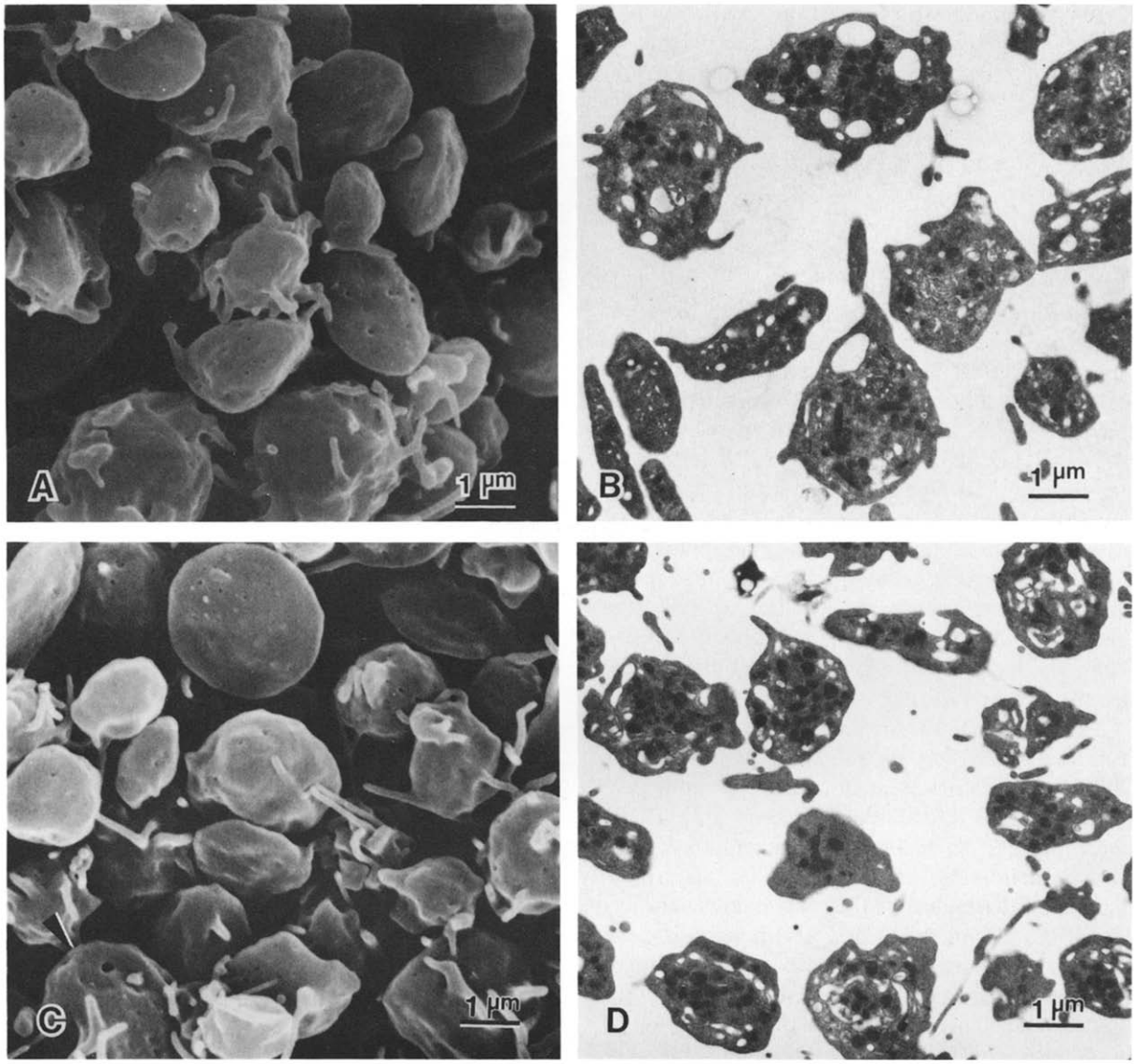
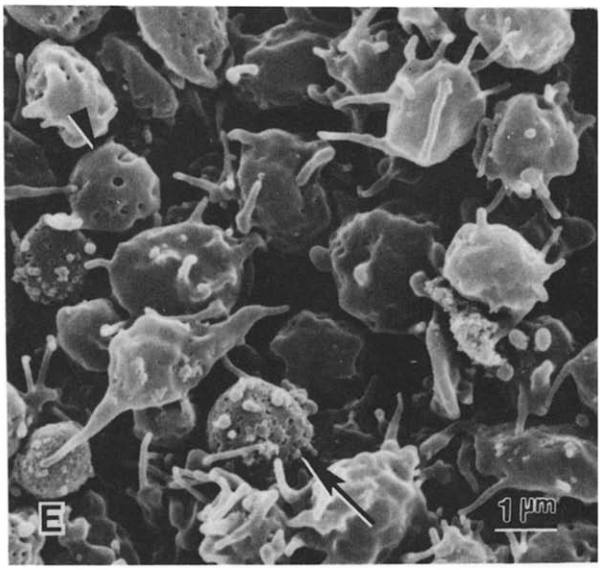
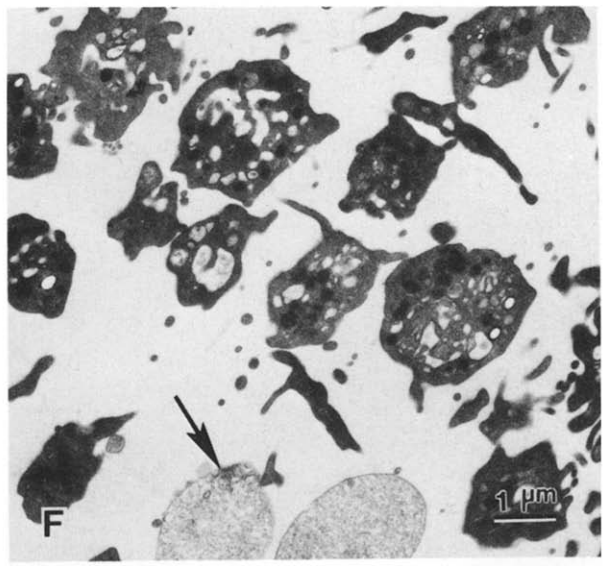


Fig. 12. Electron micrographs of phospholipase A_2-treated shape-changed platelets studied by both scanning EM ($\times 9000$ for A, C, E) and transmission EM ($\times 9000$ for B, D, F). Details of experiments are given in the Experimental procedures. The shape-changed platelets (A and B), in the presence of 10 μM indomethacin, were treated with the enzyme for 50 min (C and D) and 130 min (E and F). Arrows indicate the lysed cells, while the arrow-heads indicate the cells with dilated SCOCS (surface-connected open canalicular system) holes on the cell surface.

value of PI in the membrane outer leaflet obtained in this study. However, it is improbable that the high value of PC in the outer leaflet is due exclusively to the flip-flop of PC induced by the basic phospholipase A_2. In the experiments by using ^{32}P-labeled platelets as substrates, the increase of the radioactivity in lyso-PC occurred only after 50 min incubation in all three physiological types of platelet membrane (Figs. 6, 8, 11). By that time, the first hydrolytic phase has almost reached plateau. The distribution of phospholipid on the two sides of the platelet plasma membrane may not be equal. To balance the bilayer structure, the platelet plasma membrane may possess an asym-





metric distribution of cholesterol, present in the platelet membrane with a molar ratio to phospholipids of 74% [26]. Another possibility is that the membrane inner leaflet is filled with more proteins than the outer one to compensate the vacant spacing.

This study confirms that thrombin induces the exposure of PS on the platelet membrane surface [3,7,8]. PS is not exposed on the shape-changed platelet membrane surface. Bevers et al. [8] have suggested that the mechanism of PS exposure is by the flip-flop of phospholipid in the plasma membrane. The mechanism may arise during platelet secretion.

This study suggests that the basic phospholipase A_2 of *N. nigricollis* may be a useful probe to analyze the asymmetric distribution of membrane phospholipids. It indicates that PS is not exposed on the membrane surface of the shape-changed platelet.

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