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VESICULATION OF PLATELET PLASMA MEMBRANES

DILAUROYLGLYCEROPHOSPHOCHOLINE-INDUCED SHEDDING OF A PLATELET PLASMA MEMBRANE FRACTION ENRICHED IN ACETYLCHOLINESTERASE ACTIVITY

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Incubation of washed rabbit platelets with suspensions of dilauroylglycerophosphocholine resulted in the shedding of vesicles without causing any appreciable leakage of cytoplasmic marker (lactate dehydrogenase) or organelle marker ([^{14}C]serotonin). The response was dependent on incubation time, concentration of dilauroylglycerophosphocholine and reaction temperature. Vesicles were separated from platelets and exogenous dilauroylglycerophosphocholine by a series of centrifugation steps. An average diameter of vesicles was 100–200 nm on scanning electron microscopy. Vesicles were enriched 5-fold in plasma membrane marker enzyme, acetylcholinesterase, whereas specific activities of lactate dehydrogenase and intracellular membrane marker enzyme, NADH-cytochrome *c* reductase were decreased in vesicles. Protein analysis by SDS-polyacrylamide gel electrophoresis showed that actin and actin-binding protein were present, while myosin was barely detectable in vesicles. Vesicles contained all phospholipid species of intact platelets and cholesterol but almost 50% of phospholipids in vesicles was dilauroylglycerophosphocholine. The phospholipid to protein ratio in vesicles was about 6.5-times higher than in intact platelets.

Introduction

In response to a number of physiological stimuli, platelets change their shapes, aggregate and secrete granule-stored procoagulants and other constituents. The platelet plasma membrane has receptors for these stimuli, which transmit information across the membrane through stimuli-receptor interactions. For this and other reasons, much interest has been raised in the constitution and function of the platelet plasma membranes.

Several attempts have been made to obtain plasma membrane fraction of platelets. All these procedures of plasma membrane preparation include disruption of platelets, which should cause damage to the plasma membrane. Therefore, for many purposes it is an advantage to prepare plasma membrane vesicles without cell lysis by a shedding process. The shedding of vesicles occurs physiologically in normal and tumor cells [1]. Vesiculation can be induced also by chemical agents [2–4] or detergents [5,6]. Such reagents have been used for the isolation of plasma membrane vesicles from monolayer cell culture [2], thymocyte [5], *Dictyostelium discoideum* [3] and erythrocytes [4,6], but to our knowledge, vesiculation has not been

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

previously applied for the isolation of platelet plasma membranes.

Recently Ott et al. [7] showed that the incubation of human erythrocytes with dimyristoylglycerophosphocholine caused the shedding of vesicles. We have demonstrated that dilauroylglycerophosphocholine induced the shedding of vesicles from erythrocytes of various species of animals without causing any appreciable hemolysis [8]. It was suggested that the incorporation of monomers of dilauroylglycerophosphocholine into the erythrocyte membrane may trigger the shedding of vesicles. In the present study, we used dilauroylglycerophosphocholine for the isolation of platelet plasma membranes and demonstrated that vesicles which exhibit characteristic features of plasma membrane were shed from intact rabbit platelets. Some properties of the shed vesicles were characterized.

Materials and Methods

Materials. Dilauroylglycerophosphocholine, cytochrome *c* (horse heart), poly-L-lysine (approx. mol. wt. 65 000), antimycin A, bis(*p*-nitrophenyl) phosphate, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 5-Hydroxy[*side-chain*-2-^{14}C]tryptamine creatine sulfate (serotonin), 55 mCi/mmol, was obtained from the Radiochemical Centre, Amersham, U.K. Other chemicals were all of reagent grade.

Buffer. Hepes-buffered saline (140 mM NaCl/10 mM Hepes (pH 6.8)) containing 5 mM glucose and 1 mM EDTA (standard buffer) was used throughout this work unless otherwise noted.

Preparation of platelets. Platelets were obtained from rabbit blood collected in the presence of 1/10 vol. of 3.8% trisodium citrate. Citrated blood was centrifuged for 10 min at $170 \times g$ and the resulting platelet-rich plasma was recentrifuged at $645 \times g$ for 10 min. The platelet pellet was dispersed and washed twice with the standard buffer and then suspended in the same buffer. The preparation of washed platelets were carried out at room temperature.

Preparation of liposomes. Vesicles of phosphatidylcholine were prepared by suspending a dried sample of a lipid in the standard buffer and sonicating the suspension in a sonicator equipped with a microprobe (Branson Sonifier Model 200, operating at 15 W) for 10 min at 0°C. The dispersions were then centrifuged at $15\,800 \times g$ for 5 min to remove titanium particles and multilamellar vesicles, and supernatants were taken for use.

Treatment of platelets with phosphatidylcholine liposomes. An appropriate amount of a phosphatidylcholine suspension was preincubated at a given temperature for 5 min and the reaction was started by adding washed platelets to this suspension so that the platelet concentration was $5 \cdot 10^5$ cells per μl. Unless stated otherwise, the incubation temperature was 25°C and the concentration of phosphatidylcholine in the incubation mixture was 100 μM. After 10 min of incubation, reaction mixture was centrifuged at $645 \times g$ for 10 min to remove the platelets. The supernatant was further centrifuged at $30\,000 \times g$ for 30 min at 25°C in a Hitachi 65P-7 ultracentrifuge equipped with a RP65 angle rotor. The pellet of vesicles was washed twice by resuspending in the standard buffer followed by centrifugation at $30\,000 \times g$ for 30 min. The supernatant which contained the bulk of dilauroylglycerophosphocholine were discarded. The pellet obtained after the final centrifugation was resuspended in buffer and then centrifuged at $645 \times g$ for 10 min to remove contaminated platelets and resulting supernatant was taken for experiments.

Leakage of serotonin. 5 ml of platelet-rich plasma was incubated with 2 μCi of [^{14}C]serotonin for 20 min at 25°C. Then washed platelets were prepared and incubated with dilauroylglycerophosphocholine as described above. At appropriate times, the reaction mixture was centrifuged at $645 \times g$ for 10 min and an aliquot of the supernatant was analyzed for radioactivity in an Aloka LSC-900 liquid scintillation counter.

Scanning electron microscopic observations. Scanning electron microscopic observations were made essentially according to Kanaho and Fujii [9]. The pellets of vesicles obtained by centrifugation at $30\,000 \times g$ were fixed with 1% glutaraldehyde/1% osmic acid and adsorbed onto coverslips previously coated with poly-L-lysine. The sample was then subjected to serial dehydration in ethanol, critical point drying with liquid CO_2, and coating with gold. The specimen was examined with a scanning electron microscope (Hitachi S-430) at an

accelerating voltage of 20 kV.

Enzyme assays. Acetylcholinesterase was determined according to the method of Ellman et al. [10], and lactate dehydrogenase was measured by the method of Bergmeyer and Bent [11], though the standard buffer was employed instead of those used in the literatures. Bis(*p*-nitrophenyl)phosphate phosphodiesterase was determined as described by Taylor and Crawford [12]. Antimycin-insensitive NADH-cytochrome *c* reductase was determined as described by Tolbert [13].

Lipid estimations. Lipids were extracted by the method of Bligh and Dyer [14]. Individual phospholipids were separated by two-dimensional thin-layer chromatography [15]. Lipid phosphorous was estimated by the method of Ames [16]. Total cholesterol in lipid extracts was determined enzymatically according to the method of Stähler et al. [17] using a Monotest Cholesterol Kit (Boehringer, Mannheim). Fatty acids were analyzed as methyl esters by gas-liquid chromatography as described by Nakagawa and Horrocks [18].

Protein determination. Protein was measured by the method of Bensadoun and Weinstein [19] using bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out following the procedure of Laemmli [20] on a 8% resolving gel overlaid with 2.5% stacking gel.

Results

Acetylcholinesterase activity in washed rabbit platelets

Augustinsson et al. [21] demonstrated the presence of cholinesterase activity in platelets from rat, cat, rabbit, dog and horse. They concluded that platelet cholinesterase was an acetylcholinesterase. We measured acetylcholinesterase activity and lactate dehydrogenase activity, which is a cytoplasmic marker, in intact rabbit platelets and disrupted ones, which were sonicated with a microprobe (Branson Sonifier Model 200, operating at 15 W) for 1 min at 0°C. 83.2 ± 3.6% (mean of five experiments ± S.D.) of acetylcholinesterase activity of disrupted platelets could be detected in intact platelets. The repeated washing of platelets did not deplete the activity. On the other hand, only 4.6 ± 1.9% (mean of five experiments ± S.D.) of lactate dehydrogenase activity of disrupted platelets was recovered in intact platelets. The difference of these two enzyme activities in intact platelets (approx. 80%) can be regarded to reflect acetylcholinesterase which is situated on the ambient side of plasma membrane.

Exfoliation of acetylcholinesterase activity from washed platelets treated with dilauroylglycerophosphocholine

Incubation of washed platelets with dilauroylglycerophosphocholine liposomes at 25°C caused the exfoliation of acetylcholinesterase activity from platelets (Figs. 1, A and B). Acetylcholinesterase activity was exfoliated without detectable lag period. The exfoliation continued for up to 15 min without leakage of cytoplasmic marker, lactate dehydrogenase or [^{14}C]serotonin, which is an organelle marker (Fig. 1A). During incubation, aggregation of platelets was not observed. Acetylcholinesterase exfoliation was dependent on the concentration of added dilauroylglycerophosphocholine (Fig. 1B). Without dilauroylglycerophosphocholine, no acetylcholinesterase exfoliation could be observed. Total acetylcholinesterase activity in reaction mixture remained constant during all experiments.

The influence of incubation temperature on exfoliation of acetylcholinesterase activity is shown in Table I. Dilauroylglycerophosphocholine induced the exfoliation of acetylcholinesterase activity at all temperatures tested. At 10°C, the concentration of dilauroylglycerophosphocholine required for 50% exfoliation after 10 min incubation was 650 μM, while it was 60 μM at 37°C. The time required for 50% exfoliation by 100 μM dilauroylglycerophosphocholine was shortened as incubation temperature was increased. Dimyristoylglycerophosphocholine and dipalmitoylglycerophosphocholine did not induce the exfoliation of acetylcholinesterase activity even at 37°C under these conditions.

Separation of vesicles from platelets and exogenous dilauroylglycerophosphocholine

When the medium, which contained acetylcholinesterase exfoliated from platelets, was further centrifuged at 30000 × *g* for 30 min, more

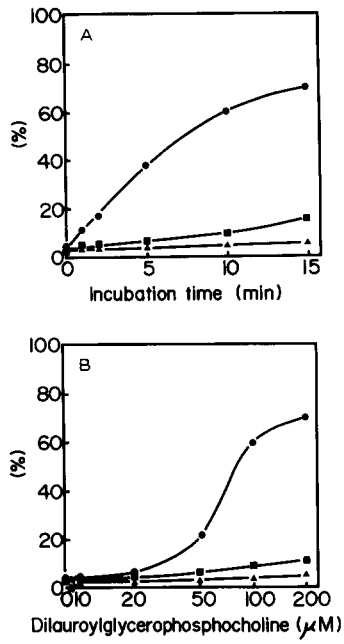

Fig. 1. Release of acetylcholinesterase activity, lactate dehydrogenase activity and [^{14}C]serotonin from platelets induced by dilauroylglycerophosphocholine at 25°C. (A) Time-course of release of acetylcholinesterase activity (●), lactate dehydrogenase activity (▲) and radioactivity of [^{14}C]serotonin (■). Washed rabbit platelets ($5 \cdot 10^5$ cells/μl) were incubated with 100 μM dilauroylglycerophosphocholine. Parameters were determined in the supernatant after the removal of platelets by centrifugation as described in Materials and Methods. For determination of 100% of each parameter, the incubation mixture was disrupted in a sonicator equipped with a microprobe (Branson Sonifier Model 200, operating at 15 W) for 1 min at 0°C. (B) The concentration dependence of release of acetylcholinesterase activity (●), lactate dehydrogenase activity (▲) and radioactivity of [^{14}C]serotonin (■) on dilauroylglycerophosphocholine. Washed rabbit platelets ($5 \cdot 10^5$ cells/μl) were incubated with various concentrations of dilauroylglycerophosphocholine for 10 min and then acetylcholinesterase activity, lactate dehydrogenase activity and radioactivity of [^{14}C]serotonin in the supernatant after the removal of platelets were determined as described in Materials and Methods.

TABLE I

EFFECT OF TEMPERATURE ON EXFOLIATION OF ACETYLCHOLINESTERASE ACTIVITY FROM PLATELETS BY TREATMENT WITH DILAUROYLGLYCEROPHOSPHOCHOLINE

Rabbit platelets ($5 \cdot 10^5$ cells/μl) were incubated with various concentrations of dilauroylglycerophosphocholine at 10°C, 25°C and 37°C. The exfoliation of acetylcholinesterase activity was determined as described in Materials and Methods. Acetylcholinesterase activity of the samples sonicated as described in the legend to Fig. 1 was taken as 100%.

Temperature (°C)	Amounts of dilauroylglycerophosphocholine required for 50% exfoliation after 10 min incubation (μM)	Time required for 50% exfoliation by 100 μM dilauroylglycerophosphocholine (min)
10	650	23
25	80	7.5
37	60	1.5

than 80% of the enzyme activity was recovered from the sediment (data not shown). Almost all enzyme activity in the sediment was retained after washing with the standard buffer. $4.99 \pm 0.11\%$ (mean of three experiments ± S.D.) of total protein in platelets was recovered in the sediment. On scanning electron microscopy, vesicles with an average diameter of 100–200 nm were observed in the sediment fraction (Fig. 2). An enzyme profile of vesicles was shown in Table II. Acetylcholinesterase and bis(*p*-nitrophenyl)phosphate phosphodiesterase [22–24] were utilized as markers for plasma membrane. Lactate dehydrogenase was assayed as a marker of cytoplasm and antimycin-insensitive NADH-cytochrome *c* reductase was utilized as a marker of intracellular membranes [25]. Vesicles were enriched 5-fold in acetylcholinesterase activity, whereas the specific activity of acetylcholinesterase in platelets incubated with dilauroylglycerophosphocholine was about 45% of that in intact platelets. In contrast, another plasma membrane marker enzyme, bis(*p*-nitrophenyl)phosphate phosphodiesterase was not enriched significantly in vesicle fraction. The specific activities of lactate dehydrogenase and NADH-cytochrome *c* reductase were significantly decreased in vesicles.

Fig. 3 shows the SDS-polyacrylamide gel separations of shed vesicles, platelets after incubation with dilauroylglycerophosphocholine and intact platelets. As compared with intact platelets, polypeptides of apparent mol. wt. 250000, which is presumably identical to actin-binding protein [26], 115000, 70000, 50000 and actin were prominent in vesicles, while myosin was barely detectable and polypeptide of apparent mol. wt. 55000 was faint in vesicles.

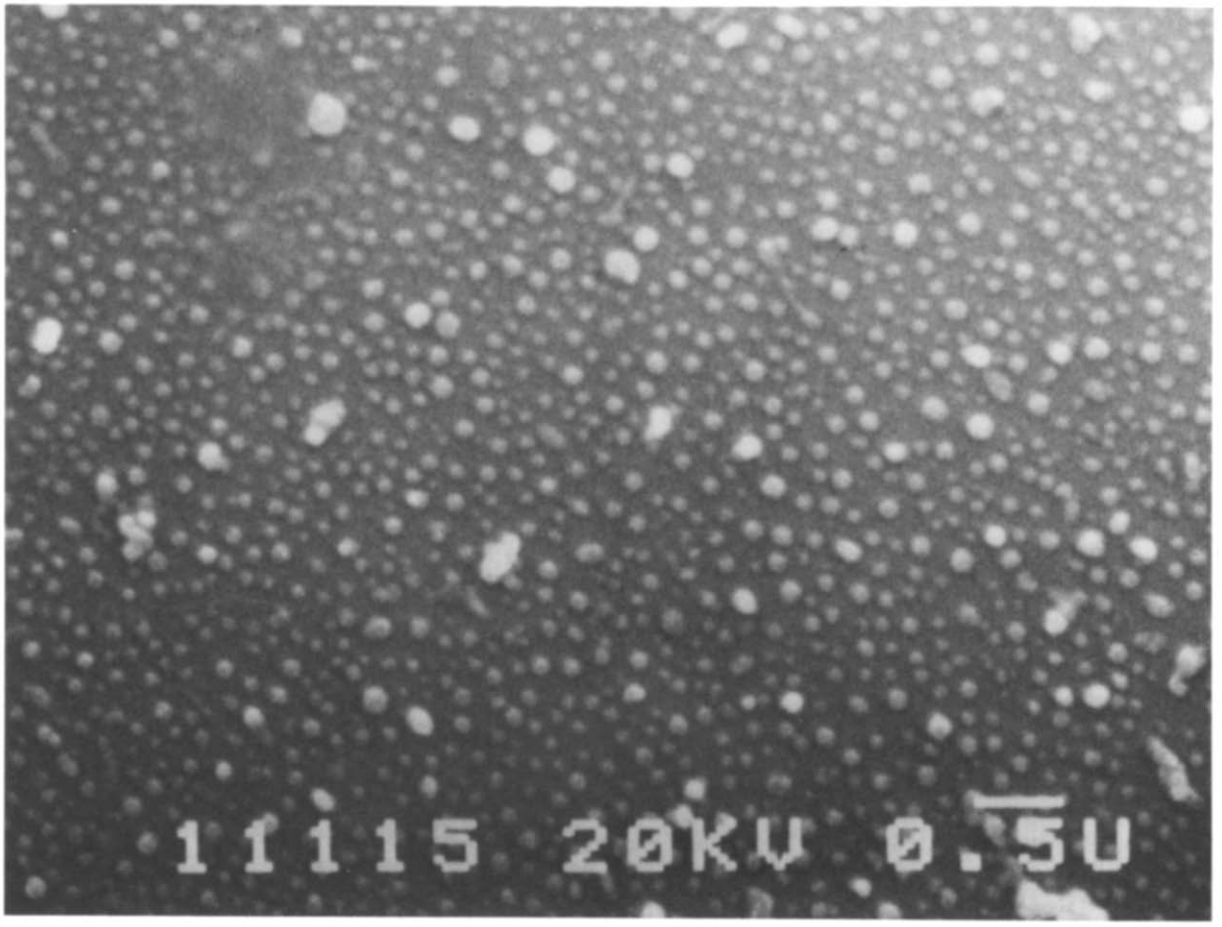

Fig. 2. Scanning electron micrograph of shed vesicles. Washed rabbit platelets were incubated with dilauroylglycerophosphocholine as described in Materials and Methods. Aliquots of the 30000 × *g* pellet were examined under an electron microscope. Bar, 0.5 μm.

Lipid composition of shed vesicles

Dilauroylglycerophosphocholine could be separated from phosphatidylcholine of platelet origin by two-dimensional thin-layer chromatography (Fig. 4). The newly appeared spot on chromatogram of extracted lipids from dilauroylgly-

TABLE II

ENZYME ACTIVITIES IN SHED VESICLES, DILAUROYLGLYCEROPHOSPHOCHOLINE-TREATED PLATELETS AND INTACT PLATELETS

Enzyme activities were determined as described in Materials and Methods in the presence of 0.1% Triton X-100. Data are expressed as nanomoles of product formed per minute per milligram protein (mean of three experiments ± S.D.).

Enzymes	Vesicles	Incubated platelets	Platelets
Acetylcholinesterase	2795 ± 795	252 ± 40	555 ± 146
BpND [a] phosphodiesterase	0.104 ± 0.009	0.087 ± 0.013	0.101 ± 0.006
Lactate dehydrogenase	565 ± 16	1304 ± 40	1475 ± 125
NADH-cyt *c* [b] reductase	12.3 ± 3.4	47.8 ± 7.8	41.3 ± 6.7

[a] BpND, bis(*p*-nitrophenyl)phosphate.
[b] cyt *c*, cytochrome *c*.

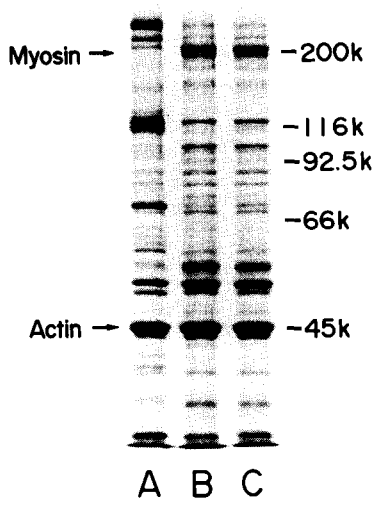

Fig. 3. SDS-polyacrylamide gel electrophoresis of shed vesicles (A), dilauroylglycerophosphocholine-treated platelets (B) and intact platelets (C). Gels were stained with Coomassie brilliant blue. The position of myosin, actin and other molecular weight standards were indicated.

cerophosphocholine-treated platelets showed the same Rf value as dilauroylglycerophosphocholine. Fatty acid analysis revealed that the fatty acid of this phospholipid was exclusively lauric acid. On the other hand, phosphatidylcholine of platelet origin in dilauroylglycerophosphocholine-treated platelets did not contain lauric acid (data not shown). These results indicate that deacylation-reacylation [28] of dilauroylglycerophosphocholine did not occur during incubation period.

The phospholipid composition of the vesicles are presented in Table III. Vesicles contained all

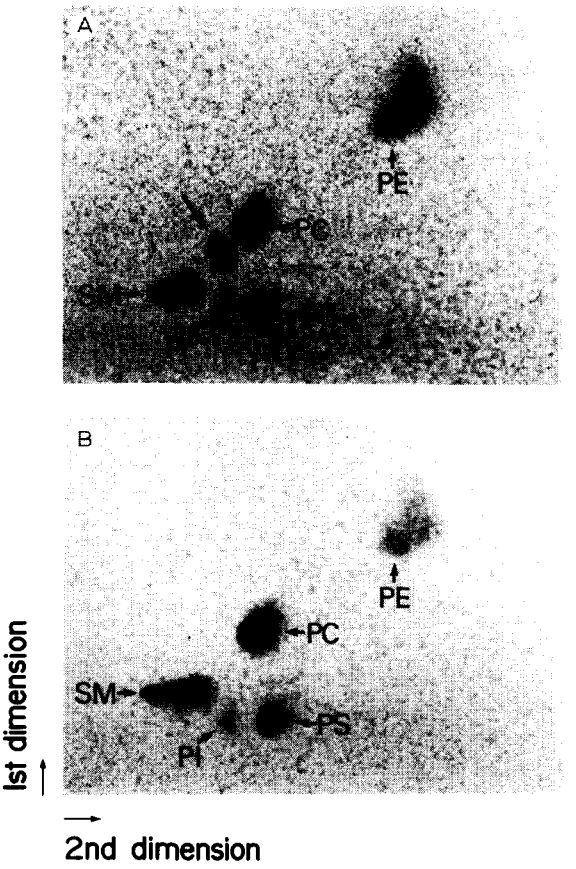

Fig. 4. Two-dimensional thin-layer chromatograph of phospholipids of dilauroylglycerophosphocholine-treated platelets (A) and intact platelets (B). Thin-layer chromatography was performed as described in Materials and Methods. Each spot was identified by Dittmer-Lester reagent [27]. The spot shown by arrow indicates dilauroylglycerophosphocholine. PE, PS, PI, SM and PC indicate phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin and phosphatidylcholine, respectively.

TABLE III

PHOSPHOLIPID COMPOSITION IN SHED VESICLES, DILAUROYLGLYCEROPHOSPHOCHOLINE-TREATED PLATELETS AND INTACT PLATELETS

Lipids were separated and determined as described in Materials and Methods. Data are expressed as mol% of total phospholipid fraction (mean of three experiments ± S.D.).

Component	Vesicles		Incubated platelets		Platelets
Phosphatidylethanolamine	16.9 ± 1.1	32.8 ± 0.9 [a]	26.0 ± 1.6	31.0 ± 1.8 [a]	30.7 ± 0.7
Phosphatidylserine	8.6 ± 0.7	16.7 ± 1.3 [a]	11.0 ± 0.6	13.1 ± 0.6 [a]	12.7 ± 2.0
Phosphatidylinositol	0.8 ± 0.1	1.6 ± 0.1 [a]	2.9 ± 0.9	3.4 ± 1.0 [a]	2.8 ± 0.6
Sphingomyelin	9.7 ± 2.0	18.8 ± 2.4 [a]	12.4 ± 1.2	14.8 ± 1.6 [a]	18.3 ± 1.8
Phosphatidylcholine	15.6 ± 1.5	30.3 ± 2.1 [a]	31.8 ± 1.4	37.8 ± 1.2 [a]	35.5 ± 1.2
Dilauroylglycerophosphocholine	48.5 ± 4.5		15.9 ± 0.9		-

[a] mol% of total phospholipid fraction except dilauroylglycerophosphocholine.

TABLE IV

CHOLESTEROL AND PHOSPHOLIPID CONTENTS OF SHED VESICLES, DILAUROYLGLYCEROPHOSPHOCHOLINE-TREATED PLATELETS AND INTACT PLATELETS

Lipids were extracted and determined as described in Materials and Methods. Data are expressed as mean of three experiments ± S.D.

Component	Vesicles	Incubated platelets	Platelets
Cholesterol (μmol/mg protein)	0.403 ± 0.029	0.085 ± 0.003	0.113 ± 0.006
Phospholipids (μmol/mg protein)	1.02 ± 0.120	0.178 ± 0.023	0.157 ± 0.014
Cholesterol/ phospholipid molar ratio	0.398 ± 0.017 0.773 ± 0.032 [a]	0.480 ± 0.044 0.571 ± 0.054 [a]	0.721 ± 0.030

[a] The amount of phospholipids except dilauroylglycerophosphocholine was used for calculation.

phospholipid species in intact platelets. In addition, about 50% of phospholipid in vesicles was dilauroylglycerophosphocholine, which was also included as much as 16% in platelets incubated with dilauroylglycerophosphocholine. When the phospholipid composition expressed as mol% of total phospholipid fraction except dilauroylglycerophosphocholine was compared between vesicles and intact platelets, a slight enrichment in phosphatidylethanolamine and phosphatidylserine and the decrease in phosphatidylinositol and phosphatidylcholine were observed in vesicles. The cholesterol and phospholipid contents and their ratios in shed vesicles, platelets after incubated with dilauroylglycerophosphocholine and intact platelets are presented in Table IV. Cholesterol to protein and phospholipid to protein ratios were increased in vesicles 3.6-times and 6.5-times, respectively, as compared with intact platelets. The cholesterol/phospholipid molar ratio decreased in the order intact platelets, dilauroylglycerophosphocholine-treated platelets, vesicles. But when phospholipids of platelet origin was used for calculation, vesicles showed the highest ratio, while dilauroylglycerophosphocholine-treated platelets the lowest.

Fatty acid compositions of phosphatidylcholine of platelet origin and phosphatidylethanolamine were compared for vesicles, dilauroylglycerophosphocholine-treated platelets and intact platelets. No significant differences were observed in fatty acid compositions among them (data not shown).

Discussion

Incubation of washed rabbit platelets with suspensions of dilauroylglycerophosphocholine resulted in the exfoliation of acetylcholinesterase activity without causing any appreciable leakage of cytoplasmic marker (lactate dehydrogenase) or organelle marker ([^{14}C]serotonin). Almost 70% of acetylcholinesterase activity in platelets was exfoliated during the reaction. Since the exfoliation of acetylcholinesterase activity occurred under non-lytic conditions, one can postulate that the exfoliated acetylcholinesterase comes from the plasma membrane. This idea is compatible with our rough estimation that approx. 80% of acetylcholinesterase activity of rabbit platelets was situated on the plasma membrane. Previously Lovette et al. [29] examined the subcellular distribution of cholinesterase activities of canine platelets. They showed only about 30% of cholinesterase activity was presented in the plasma membrane. At present, it is not known whether the discrepancy results simply from the difference of two animal species. The exfoliated activity was not the contamination of plasma cholinesterase since repeated washing of platelets did not deplete the activity.

Most of the acetylcholinesterase activity exfoliated into the medium was associated with vesicles of diameter 100–200 nm. The specific activity of antimycin-insensitive NADH-cytochrome *c* reductase was significantly decreased in vesicles. Actin was present while myosin was absent in vesicles. Vesicles were enriched in actin-binding

protein. Cholesterol and phosphatidylethanolamine were enriched in vesicles, whereas phosphatidylinositol and phosphatidylcholine were decreased. These properties of vesicles were similar to those of plasma membrane fraction N_{III} of human platelets, which was isolated by Crawford and co-workers [25,30] using high voltage free flow electrophoresis.

The preferential exfoliation of acetylcholinesterase over phosphodiesterase suggest that the shedding process was selective and that the vesicles consisted of specific domains of the plasma membrane. Similar findings of selective enrichment of plasma membrane components in vesicles were reported in monolayer cultures [31], thymocyte [5,32] and erythrocyte [8,33]. The ratio of dilauroylglycerophosphocholine to total phospholipid in vesicles was three-times higher than that in platelets incubated with dilauroylglycerophosphocholine. In a previous study [8], we reported that in erythrocytes, the ratio of dilauroylglycerophosphocholine to total phospholipid in the vesicles was almost the same as that observed in the dilauroylglycerophosphocholine-treated erythrocyte fraction. These findings suggest that vesicles were shed from portions of the plasma membranes in which dilauroylglycerophosphocholine were incorporated. Assuming that the vesiculation of platelet occurs in the same manner as that of erythrocytes, only one third of total platelet membranes was considered to be susceptible to dilauroylglycerophosphocholine. The extremely slow rate of the flip-flop of dilauroylglycerophosphocholine [34] suggest that dilauroylglycerophosphocholine could not be incorporated into intracellular membranes across the plasma membrane. Chap et al. [35] estimated that 63% of platelet phospholipid was located in the plasma membrane. Therefore, our results suggest that only half the region of platelet plasma membranes was available for incorporation of dilauroylglycerophosphocholine.

The vesicles shed from platelets possessed several properties similar to those of the vesicles shed from erythrocytes by treatment with dilauroylglycerophosphocholine [8]; that is, the diameter of 100–200 nm, the enrichment of acetylcholinesterase activity, the increase of phospholipid to protein ratio and the temperature dependence of the reaction. Like vesiculation of erythrocytes, the exfoliation of acetylcholinesterase activity from platelets by dilauroylglycerophosphocholine was inhibited by serum albumin or incorporation of cholesterol into liposomes of dilauroylglycerophosphocholine (Kobayashi, T., unpublished observation).

The vesiculation of erythrocytes occurs under various conditions, such as depletion of endogenous ATP [4,33] or reoxygenation of sickled erythrocytes [36] and is considered to be involved in the process of erythrocyte aging [33]. Further experiments are required to elucidate whether vesiculation of platelets occurs physiologically.

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