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Inhibition of platelet aggregation by synthetic phosphatidylcholines: possible involvement of vesiculation of platelet plasma membranes

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Synthetic phosphatidylcholines inhibited thrombin-induced aggregation of rabbit platelets. The inhibitory effect of the phosphatidylcholines increased with an increase in the chain-length of the constituent fatty acids up to 12, and then decreased, and $C_{14:0}$PC and $C_{16:0}$PC did not inhibit platelet aggregation. The activity of synthetic phosphatidylcholines as to induction of vesiculation of platelet plasma membranes (Kobayashi, T., Okamoto, H., Yamada, J.-I., Setaka, M. and Kwan, T. (1984) Biochim. Biophys. Acta 778, 210–218) and the inhibitory effect of these phosphatidylcholines on platelet aggregation showed the same dependence on the constituent fatty acids of the phosphatidylcholines. The amounts of phosphatidylcholines required for 50% inhibition of platelet aggregation correspond very well to those required for 15% exfoliation of acetylcholinesterase activity, suggesting that there is a close relationship between platelet aggregation and vesiculation of the platelet plasma membrane. The possible mechanism of inhibition of platelet aggregation by synthetic phosphatidylcholines is discussed.

Introduction

Platelet activation is a sequence of morphological and functional changes, which are triggered by various stimulatory agents. The platelet plasma membrane plays an important role in the reception of external stimuli, in the transmission of the messages into the cell and in the execution of the platelet response.

Various amphiphilic substances are known to affect platelet functions. Although the mechanism of the action of these agents is obscure, it is very likely that the effect of most of the amphiphilic substances is on plasma membrane organization.

Previously, Kerr et al. [1] investigated the effects of phospholipids and free fatty acids on platelet aggregation and concluded that phosphatidylcholine did not affect platelet aggregation induced by ADP. Recently, Juliano et al. [2] showed that liposomes composed of egg yolk phosphatidylcholine or of a photopolymerizable phosphatidylcholine derivative did not affect platelet aggregation by ADP or thrombin. The effect of synthetic phosphatidylcholines on platelet aggregation has not been investigated previously. Among synthetic phosphatidylcholines, phosphatidylcholines with a short fatty-acyl chain, such as dilauroylglycerophosphocholine ($C_{12:0}$PC), have

Abbreviations: $C_{8:0}$PC, dioctanoylglycerophosphocholine; $C_{9:0}$PC, dinonanoylglycerophosphocholine; $C_{10:0}$PC, didecanoylglycerophosphocholine; $C_{11:0}$PC, diundecanoylglycerophosphocholine; $C_{12:0}$PC, dilauroylglycerophosphocholine; $C_{13:0}$PC, ditridecanoylglycerophosphocholine; $C_{14:0}$PC, dimyristoylglycerophosphocholine; $C_{16:0}$PC, dipalmitoylglycerophosphocholine; PAF-acether, platelet-activating factor.

similar properties to those of peroxidized natural phosphatidylcholines [3].

Recently, we have demonstrated that incubation of washed rabbit platelets with suspension of $C_{12:0}$PC resulted in the shedding of vesicles which exhibit characteristic features of the plasma membrane without any appreciable leakage of cytoplasmic marker or organelle marker [4]. In the present study, we examined the effects of various synthetic phosphatidylcholines, including $C_{12:0}$PC, on platelet aggregation and the results were compared with the activity of these phosphatidylcholines as to induction of plasma membrane vesiculation.

Materials and Methods

Materials. $C_{9:0}$PC, $C_{11:0}$PC, $C_{13:0}$PC and platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) were purchased from Avanti Polar-Lipids, Birmingham, AL, U.S.A. $C_{8:0}$PC, $C_{10:0}$PC, $C_{12:0}$PC, $C_{14:0}$PC, $C_{16:0}$PC and bovine serum albumin (essentially fatty acid free) were obtained from Sigma, St. Louis, MO, U.S.A. All lipid preparations showed single spots on silica-gel thin-layer chromatograms. Thrombin (bovin plasma, 50 NIH units/mg) was obtained from Parke-Davis through Sankyo, Tokyo, Japan. 5-Hydroxy[*side-chain*-2-^{14}C]tryptamine creatine sulfate (serotonin), 55 mCi/mmol, was obtained from the Radiochemical Centre, Amersham, U.K. All other chemicals were of reagent grade.

Preparation of platelets. Platelets were obtained from rabbit blood anticoagulated with EDTA. Washed platelets suspended in Tyrode's solution, pH 7.4, were prepared according to the method of Ardlie et al. [5]. All washing and suspending media contained 0.35% bovine serum albumin.

Preparation of phospholipid vesicles. Phosphatidylcholine vesicles were prepared by suspending a dried sample of the lipid in Tyrode's solution without bovine serum albumin and sonicating the suspension in a sonicator equipped with a microprobe (Branson Sonifier Model 200, operating at 15 W) for 10 min above the phase-transition temperature of the phospholipid. The suspension was then centrifuged at $15\,800 \times g$ for 5 min to remove titanium particles and multilamellar vesicles, and then the supernatants were taken and used after the addition of 0.35% bovine serum albumin.

Platelet aggregation. A platelet suspension ($5 \cdot 10^5$ cells/μl) was preincubated with phosphatidylcholine for 5 min at 37°C. Then, thrombin or PAF-acether was added and platelet aggregation was measured with an aggregometer (RAM-11, Rikadenki Kogyo, Co., Tokyo, Japan). The effects of reagents on aggregation were expressed as the maximum aggregation with the reagents relative to that without reagents.

Platelet release reaction. 5 ml of platelet-rich plasma was incubated with 2 μCi [^{14}C]serotonin for 30 min at 37°C. Then, washed platelets were prepared and incubated with phosphatidylcholine as described above. 1 min after the addition of thrombin, secretion was stopped with 1.5% formaldehyde [6]. The reaction mixture was centrifuged at $15\,800 \times g$ for 1 min and an aliquot of the supernatant was analyzed for radioactivity with an Aloka LSC-900 liquid scintillation counter.

Measurement of vesiculation of platelet plasma membranes. Vesiculation of platelets was assayed by measuring the exfoliation of acetylcholinesterase activity from platelets as described previously [4].

Results

Effect of $C_{12:0}$PC on platelet aggregation

$C_{12:0}$PC inhibited both thrombin- and PAF-acether-induced aggregation of platelets in a concentration-dependent manner (Fig. 1). Platelet aggregation by both stimulatory agents was not affected by pretreatment with $C_{12:0}$PC at less than 20 μM. By examining various concentrations of $C_{12:0}$PC, 41 μM was determined to be the average value for 50% inhibition of maximum aggregation of platelets by 0.1 U/ml of thrombin. Unlike lysophosphatidylcholine or chlorpromazine [7], the inhibitory effect of $C_{12:0}$PC was not abolished by washing the phospholipid-treated platelets with bovine serum albumin-containing Tyrode's solution. Instead, the platelets were lysed by washing. Fig. 2 illustrates the effect of timing of the addition of $C_{12:0}$PC on platelet aggregation. Apparently, about 1 min incubation was required for maximum inhibition. On the other hand, platelet aggregation was not affected when $C_{12:0}$PC was added after the challenge with thrombin. Fig. 3 shows that the platelet-release reaction was also

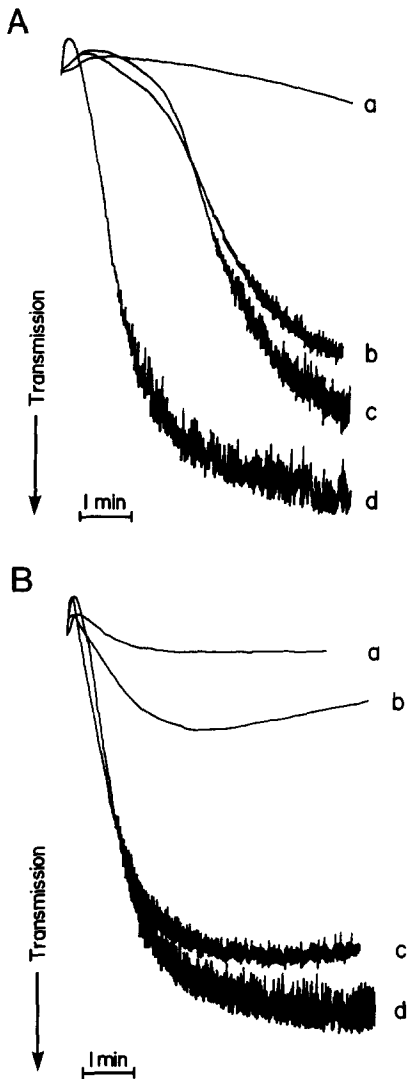

Fig. 1. Effect of $C_{12:0}PC$ on thrombin- or PAF-acether-induced platelet aggregation. Platelets were treated with 50 μM (a), 40 μM (b), 30 μM (c) and 0 μM (d) $C_{12:0}PC$ at 37°C for 5 min, and then stimulated by adding 0.1 U/ml thrombin (A) or 10^{-8} M PAF-acether (B) at 37°C under continuous stirring.

inhibited by pretreatment with $C_{12:0}PC$. 23 μM $C_{12:0}PC$ was required for 50% inhibition of [^{14}C]serotonin secretion from platelets.

Possible relationship between platelet aggregation and vesiculation of platelet plasma membranes

Incubation of washed platelets with $C_{12:0}PC$ caused the exfoliation of acetylcholinesterase activity from the platelets [4]. Acetylcholinesterase exfoliation was dependent on the concentration of $C_{12:0}PC$ added (Fig. 4). Exfoliation of acetylcholinesterase occurred without lysis of platelets, even when the platelets were incubated

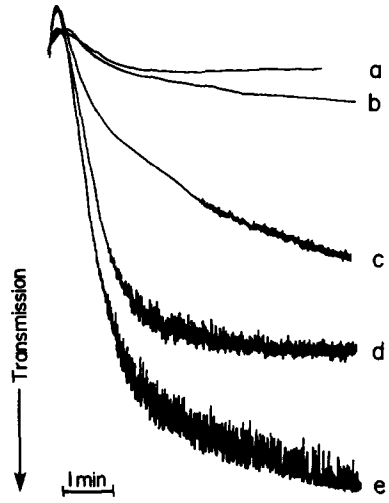

Fig. 2. Effect of timing of $C_{12:0}PC$ addition to a platelet suspension on thrombin-induced platelet aggregation. 50 μM $C_{12:0}PC$ was added to a platelet suspension 1 min before (a), 45 s before (b), 30 s before (c), at the same time as (d), and 1 min after (e) the addition of 0.1 U/ml thrombin at 37°C. Platelet aggregation was measured as described in the legend to Fig. 1.

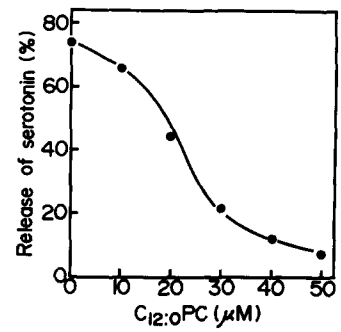

Fig. 3. Effect of $C_{12:0}PC$ on the release of [^{14}C]serotonin from platelets. [^{14}C]Serotonin-labelled platelets were incubated with various concentrations of $C_{12:0}PC$ at 37°C for 5 min, and then 0.1 U/ml thrombin was added. After 1 min incubation at 37°C, each reaction mixture was centrifuged and radioactivity of the supernatant was measured as described in Materials and Methods. For determination of 100% release, the incubation mixture was disrupted in a sonicator equipped with a microprobe (Branson Sonifier Model 200, operating at 15 W) for a min at 0°C.

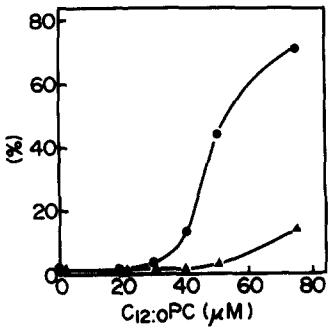

Fig. 4. Exfoliation of acetylcholinesterase activity from platelets by treatment with $C_{12:0}$PC. Platelet suspension ($5 \cdot 10^5$ cells/μl) were incubated with various concentrations of $C_{12:0}$PC at 37°C for 5 min, and then the exfoliation of acetylcholinesterase activity (●) and the release of lactate dehydrogenase activity (▲) were determined as described in Materials and Methods. For determination of 100% of each parameter, the incubation mixture was disrupted as described in the legend to Fig. 3.

with 50 μM $C_{12:0}$PC, the concentration at which platelet aggregation was completely inhibited. The reaction was completed around 1 min (data not shown).

Since the amount of $C_{12:0}$PC required for 50% inhibition of platelet aggregation corresponds very well to that of $C_{12:0}$PC for 15% exfoliation of acetylcholinesterase activity, we next examined this correspondency for various phosphatidylcholines as shown in Table I. The inhibitory effects of phosphatidylcholines on platelet aggregation increased with an increase in chain-length of the constituent fatty acids up to 12, and then decreased, and $C_{14:0}$PC and $C_{16:0}$PC did not inhibit platelet aggregation by thrombin. The activity of various phosphatidylcholines as to induction of vesiculation of platelet plasma membranes also showed the same structure dependency. The amounts of phosphatidylcholines required for 50% inhibition of platelet aggregation and those required for 15% exfoliation of acetylcholinesterase activity were almost the same.

TABLE I

EFFECTS OF VARIOUS PHOSPHATIDYLCHOLINES ON THROMBIN-INDUCED PLATELET AGGREGATION AND ON EXFOLIATION OF ACETYLCHOLINESTERASE ACTIVITY FROM PLATELETS

Platelet suspensions ($5 \cdot 10^5$ cells/μl) were incubated with various phosphatidylcholines for 5 min, and then platelet aggregation by 0.1 U/ml thrombin and exfoliation of acetylcholinesterase activity from the platelets were measured as described in the legends to Figs. 1 and 4.

Phosphatidylcholine	Amount required for 50% inhibition of aggregation (μM)	Amount required for 15% exfoliation of acetylcholinesterase activity (μM)
$C_{8:0}$PC	400	420
$C_{9:0}$PC	240	200
$C_{10:0}$PC	123	120
$C_{11:0}$PC	68	72
$C_{12:0}$PC	41	42
$C_{13:0}$PC	405	400
$C_{14:0}$PC	> 500	> 500
$C_{16:0}$PC	> 500	> 500

Discussion

Synthetic phosphatidylcholines inhibited thrombin-induced aggregation of rabbit platelets. The inhibitory effects of phosphatidylcholines increased with an increase in the chain-length of the constituent fatty acids up to 12, and then decreased, and $C_{14:0}$PC and $C_{16:0}$PC did not inhibit platelet aggregation at all. Nishizawa and Mustard [8] showed that synthetic phosphatidylserines inhibit collagen-induced platelet aggregation in pig citrated platelet-rich plasma. The extent of inhibition was related to the chain-length of the constituent fatty acids and the maximum effect was observed with $C_{10:0}$-phosphatidylserine. $C_{8:0}$- and $C_{12:0}$-phosphatidylserines had a slight effect and $C_{2:0}$-, $C_{6:0}$-, $C_{14:0}$- and $C_{16:0}$-phosphatidylserines were inactive. They also showed that $C_{10:0}$-phosphatidylserine inhibited thrombin- and collagen-induced aggregation of washed rabbit platelets. The order of synthetic phosphatidylserines as to inhibition of platelet aggregation is slightly different from that of phosphatidylcholines observed in the present study, since $C_{12:0}$PC showed the maximum inhibitory effect. Reman et al. [9] studied the hemolytic action of a series of synthetic phosphatidylcholines. Among phosphatidylcholines with fatty-acyl chains of 8–12 C atoms examined, $C_{11:0}$PC showed the highest activity and $C_{12:0}$PC the least. Our results indicate that the order as to the inhibitory effects of synthetic phosphatidylcholines on platelet aggregation

is different from that for hemolytic activity of phosphatidylcholines.

Various amphiphilic substances such as alcohols [10,11], fatty acids [1,12–14] and lysophosphatidylcholines [7,15–17] were also investigated as to their effects on platelet aggregation. It has been suggested that the effects of such amphiphilic substances on platelets are related to the changes in the membrane organization of platelets induced by these amphiphiles [7,11,14].

The inhibitory effects of various phosphatidylcholines on platelet aggregation and the activity of these phosphatidylcholines as to induction of vesiculation of platelet plasma membranes showed the same dependence on the constituent fatty acids of the phosphatidylcholines. The amount of phosphatidylcholine required for 50% inhibition of platelet aggregation was almost the same as that required for 15% exfoliation of acetylcholinesterase activity. About 1 min incubation was required for the maximum inhibition of platelet aggregation by $C_{12:0}PC$, and vesiculation of platelet plasma membranes was also completed around 1 min. These results suggest that there is a close relationship between platelet aggregation and vesiculation of platelet plasma membranes.

Although the molecular mechanism of inhibition of platelet aggregation due to vesiculation of plasma membranes is obscure, the following facts should be noted. (1) The shedding process is selective and the vesicles are composed of specific domains of the plasma membrane [4]. Although thrombin and PAF-acether are considered to stimulate platelets via different membrane receptors [18,19], $C_{12:0}PC$ inhibited platelet aggregation by both thrombin and PAF-acether at the same concentration. (2) The release of [^{14}C]serotonin from platelets was inhibited by a lower concentration of $C_{12:0}PC$ compared to that required for inhibition of platelet aggregation. The release reaction was considerably inhibited by the addition of 20 μM $C_{12:0}PC$, while the release of acetylcholinesterase activity from platelets was not observed with this concentration. Therefore, the possibility seems to be rather low that the inhibition of platelet aggregation by synthetic phosphatidylcholines is due to the exfoliation of plasma membrane receptors for stimulatory agents.

There is considerable evidence that membrane-cytoskeletal interactions are important in platelet functions [20–23]. Vesicles were enriched in actin and actin-binding protein [4]. This suggests that a rearrangement of the cytoskeletal assembly occurs in platelets prior to vesiculation. Recently, Carrol and Cox [24] reported that the effect of lysophosphatidylcholines on platelet aggregation was associated with a change in the cytoskeletal assembly in platelets. It is likely that reorganization of the platelet cytoskeleton which leads to vesiculation causes the inhibition of platelet aggregation. The mode of interaction of phosphatidylcholines with the platelet plasma membrane seems, however, to be different from that of lysophosphatidylcholines, since the inhibitory effect of $C_{12:0}PC$ on platelet aggregation was not abolished by washing the phospholipid-treated platelets with bovine serum albumin-containing Tyrode's solution.

Our previous paper [4] showed the presence of a 116 kDa band, which presumably represents the glycoprotein IIb-IIIa complex [25], in the vesicles. Several lines of evidence suggest that glycoproteins IIb and IIIa are important in fibrinogen binding to platelets [26–28] and in platelet aggregation [29–31]. It is possible to speculate about the cause of the aggregation defect in term of the lack of components of the fibrinogen receptor, glycoprotein IIb-IIIa complex. The precise mechanism of the effect of synthetic phosphatidylcholines on platelet function should be further examined.

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