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Effects of chlorpromazine and other calmodulin antagonists on phosphatidylcholine-induced vesiculation of platelet plasma membranes

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Dilauroylglycerophosphocholine ($C_{12:0}$PC)-induced vesiculation of platelet plasma membranes (Kobayashi, T., Okamoto, H., Yamada, J.-I., Setaka, M. and Kwan, T. (1984) Biochim. Biophys. Acta 778, 210–218; Kobayashi, T., Yamada, J.-I., Satoh, N., Setaka, M. and Kwan, T. (1985) Biochim. Biophys. Acta 817, 307–312) was inhibited by chlorpromazine. Preincubation of platelets with chlorpromazine was required for inhibition but incorporation of chlorpromazine into $C_{12:0}$PC liposomes was not necessary for it, indicating that the observed inhibition of vesiculation was mainly due to the effect of chlorpromazine on platelets and not that on liposomes. The change in platelet membrane fluidity caused by chlorpromazine was not the cause of inhibition of vesiculation. The inhibition of vesiculation by various other calmodulin antagonists was also observed. The inhibitory activities of these calmodulin antagonists and chlorpromazine correspond very well to their abilities to bind to calmodulin. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) inhibited vesiculation but a structural analogue of it, *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), had no inhibitory activity. These results suggest the involvement of calmodulin in membrane vesiculation.

Introduction

The shedding of membrane vesicles from the cell surface has been shown to occur in normal and tumor cells both in vivo and in vitro [1]. The process may be important in tumor-associated blood coagulation [2], in protection of a tumor from host immune rejection [3,4], in maturation of red cells [5] and in red cell aging [6]. Chemical-induced membrane shedding has also been demonstrated for a number of cell types [7–13]. Although membrane shedding occurs widely, little is known of the events that cause shedding of membrane vesicles.

In our previous papers [14,15], we showed that dilauroylglycerophosphocholine ($C_{12:0}$PC) induced the shedding of plasma membrane vesicles from washed rabbit platelets. The vesicles were enriched in actin, actin-binding protein and the plasma membrane marker enzyme, acetylcholinesterase [14]. In the present study, we examined the effect of chlorpromazine and other calmodulin antagonists, such as trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), on vesiculation of platelet plasma membranes induced by $C_{12:0}$PC. These calmodulin antagonists inhibited the shedding of membrane vesicles in a concentration-dependent manner. The possible involvement of calmodulin in the vesiculation is discussed.

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Abbreviations: $C_{12:0}$PC, dilauroylglycerophosphocholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Materials. $C_{12:0}$PC, chlorpromazine hydrochloride, trifluoperazine dihydrochloride, imipramine hydrochloride, lidocaine, colchicine, cytochalasin B, 5-doxylstearic acid and 16-doxylstearic acid were purchased from Sigma, St. Louis, MO, U.S.A. *N*-(6-Aminohexyl)-1-naphthalenesulfonamide (W-5) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) were from Seikagaku Kogyo, Tokyo, Japan. Diazepam was obtained from Wako, Osaka, Japan.

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

Preparation of platelets and phospholipid vesicles. Washed rabbit platelets and $C_{12:0}$PC vesicles were prepared as described previously [14]. For preparation of chlorpromazine-incorporated $C_{12:0}$PC liposomes, a chloroform solution of $C_{12:0}$PC and chlorpromazine was evaporated to dryness. Then, the dried sample was suspended in the buffer and sonicated as described previously [14].

Measurement of vesiculation of platelet plasma membranes. Vesiculation of platelets was assayed by measuring the exfoliation of acetylcholinesterase activity from pletelets as described previously [14].

ESR measurement. A spin probe, 40 μM, was added to a platelet suspension ($5 \cdot 10^5$ cells/μl) and the mixture was incubated for 10 min at 37°C. Then, the suspension was cooled at 25°C for 5 min and chlorpromazine was added. After 20 min incubation at 25°C, the suspension was centrifuged at 645 × *g* for 10 min and the ESR spectrum of the pellet was measured at 25°C with a JEOL JM-FE spectrometer.

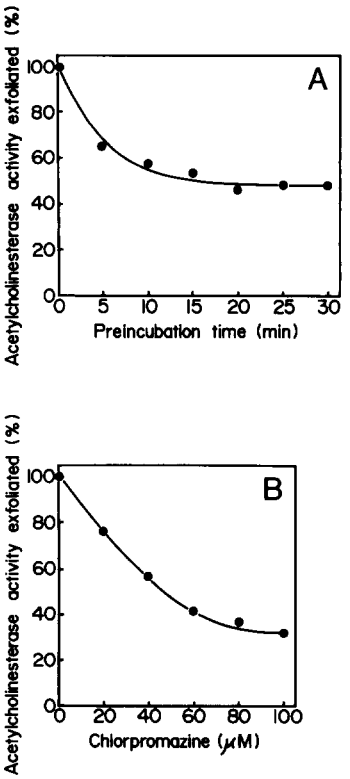

Fig. 1. Effect of chlorpromazine on $C_{12:0}$PC-induced vesiculation of platelet plasma membranes. (A) Washed rabbit platelets ($5 \cdot 10^5$ cells/μl) were preincubated with 50 μM chlorpromazine at 25°C. At appropriate intervals, 100 μM $C_{12:0}$PC was added and after 10 min incubation, exfoliation of acetylcholinesterase activity from platelets was assayed as described under Materials and Methods. The value without preincubation was taken as 100%. (B) Washed rabbit platlets ($5 \cdot 10^5$ cells/μl) were preincubated with various concentrations of chlorpromazine for 20 min at 25°C. Then, 100 μM $C_{12:0}$PC was added and after 10 min incubation, exfoliation of acetylcholinesterase activity from platelets was determined. The value without chlorpromazine was taken as 100%.

Results and Discussion

Effect of chlorpromazine on $C_{12:0}$PC-induced vesiculation of platelet plasma membranes

Preincubation of washed platelets with chlorpromazine inhibited $C_{12:0}$PC-induced exfoliation of acetylcholinesterase activity from platelets (Fig. 1A). The degree of inhibition was dependent on the preincubation time and reached a plateau within 20 min. Chlorpromazine did not decrease the acetylcholinesterase activity in the concentration range tested. Although chlorpromazine was reported to induce the extraction of membrane components from erythrocytes [16], it did not induce the exfoliation of acetylcholinesterase activity from platelets under our experimental conditions. The phospholipid and protein contents of the 30000 × *g* pellet, which is the vesicle fraction, decreased coincidently with the decrease in acetylcholinesterase activity (data not shown). The results presented above indicate that chlorpromazine inhibits vesiculation of platelet plasma membranes. The incorporation of $C_{12:0}$PC into plate-

lets was not affected significantly by preincubation of the platelets with chlorpromazine (data not shown). The inhibitory effect of chlorpromazine on vesiculation was abolished by washing the chlorpromazine-treated platelets with buffer. Chlorpromazine inhibited the exfoliation of acetylcholinesterase activity in a concentration-dependent manner (Fig. 1B). 46 μM chlorpromazine was required for 50% inhibition.

Chlorpromazine is known to affect the physicochemical properties of phospholipid vesicles [17–19]. In Fig. 2, the effect of chlorpromazine incorporated into $C_{12:0}$PC liposomes on vesiculation was examined. Although the incorporation of chlorpromazine into $C_{12:0}$PC liposomes depressed the exfoliation of acetylcholinesterase activity, the degree of inhibition was much less than that shown in Fig. 1B, for which platelets were preincubated with chlorpromazine. This result, together with the finding that preincubation of platelets with chlorpromazine was required for the inhibition of the exfoliation of acetylcholinesterase activity, suggests that the observed inhibition of vesiculation by chlorpromazine was mainly due to the effect of chlorpromazine on the platelets.

The result in zero-preincubation time in Fig. 1A, in which platelets were incubated simultaneously with chlorpromazine (50 μM) and $C_{12:0}$PC for 10 min, contrasts with the result for 50 μM chlorpromazine in Fig. 2, because approx. 20% inhibition was induced in the latter experiment. If the rate of incorporation of $C_{12:0}$PC to platelet membrane is much faster than that of chlorpromazine in free form, the inhibition effect of chlorpromazine would not be observed as in Fig. 1A in which chlorpromazine was added to platelet suspension simultaneously with $C_{12:0}$PC. If the mechanisms of inhibition of vesiculation by liposome-incorporated chlorpromazine are similar to that by free chlorpromazine, Fig. 2 indicates that the rate of incorporation of chlorpromazine in liposomes to platelet membrane should be faster than that of free chlorpromazine, since incorporation of chlorpromazine into $C_{12:0}$PC liposomes depressed the exfoliation of acetylcholinesterase activity. Another possibility to explain the result in Fig. 2 is that the incorporation of chlorpromazine into $C_{12:0}$PC liposomes decreased the rate of transfer of $C_{12:0}$PC to the platelets as observed in cholesterol or egg yolk phosphatidylcholine [20].

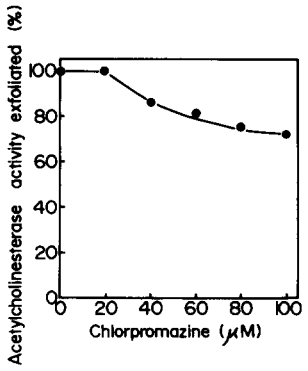

Fig. 2. Effect of chlorpromazine incorporated into $C_{12:0}$PC liposomes on vesiculation of platelet plasma membranes. Washed rabbit platelets ($5 \cdot 10^5$ cells/μl) were incubated with $C_{12:0}$PC liposomes containing various concentrations of chlorpromazine. After 10 min incubation at 25°C, the exfoliation of acetylcholinesterase activity from platelets was determined as described under Materials and Methods. The value without chlorpromazine was taken as 100%.

The membrane fluidity of platelets is affected by chlorpromazine [21]. We investigated the effect of chlorpromazine on spectral parameters of fatty acid spin labels incorporated into platelet membranes (Table I). The spectral parameters of spin probes were not affected by 50 μM chlorpromazine. Addition of 100 μM chlorpromazine decreased both $2T_{\parallel}$ of 5-doxylstearic acid and h_0/h_{-1} of 16-doxylstearic acid, indicating an increase in membrane fluidity [22,23]. Since vesiculation was inhibited significantly by 50 μM chlorpromazine, the increase in membrane fluidity is not the cause of the inhibition of vesiculation.

TABLE I

EFFECT OF CHLORPROMAZINE ON SPECTRAL PARAMETERS OF SPIN PROBES

$2T_{\parallel}$, outer hyperfine splittings; h_0, mid-field line height; h_{-1}, high-field line height.

Concentration of chlorpromazine (μM)	$2T_{\parallel}$ of 5-doxylstearic acid (gauss)	h_0/h_{-1} of 16-doxylstearic acid
0	57.4	2.43
50	57.2	2.45
100	54.4	2.26

Effects of various calmodulin antagonists on $C_{12:0}$PC-induced vesiculation of platelet plasma membranes

Studies have shown that certain phenothiazines, such as trifluoperazine and chlorpromazine, can bind to calmodulin and block various biological functions of Ca^{2+}-calmodulin complexes [24]. In Table II, the abilities of various drugs to inhibit the exfoliation of acetylcholinesterase activity and their binding to calmodulin are compared. The inhibitory activities of trifluoperazine, chlorpromazine, imipramine and diazepam correspond very well to their abilities to bind to calmodulin, suggesting the involvement of calmodulin in the process of vesiculation. We also examined two derivatives of naphthalenesulfonamide, *N*-(6-aminohexyl)-1-naphthalenesulfonamide and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide. *N*-(6-Aminohexyl)-1-naphthalenesulfonamide and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide are similar in both structure and hydrophobic properties, but *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide is much mor effective as a calmodulin antagonist [26]. As shown in Fig. 3, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide but not *N*-(6-aminohexyl)-1-naphthalenesulfonamide inhibited the exfoliation of acetylcholinesterase activity. Acetylcholinesterase activity itself was not affected by either reagent.

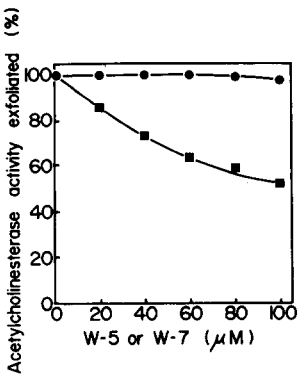

Fig. 3. Effects of *N*-(6-aminohexyl)-1-naphthalenesulfonamide and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide on $C_{12:0}$PC-induced vesiculation of platelet plasma membranes. Platelet suspensions ($5 \cdot 10^5$ cells/μl) were preincubated with various concentrations of *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) (●) or *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (■) for 20 min at 25°C. Then, 100 μM $C_{12:0}$PC was added and after 10 min incubation, exfoliation of acetylcholinesterase activity from platelets was assayed. The value without an inhibitor was taken as 100%.

TABLE II

EFFECTS OF VARIOUS DRUGS ON $C_{12:0}$PC-INDUCED VESICULATION OF PLATELET PLASMA MEMBRANES

Platelet suspensions ($5 \cdot 10^5$ cells/μl) were preincubated with various drugs for 20 min at 25°C. Then, 100 μM $C_{12:0}$PC was added and after 10 min incubation, exfoliation of acetylcholinesterase activity from platelets was determined.

Drug	Amount required for 50% inhibition of exfoliation of cholinesterase activity (μM)	Calcium specific binding of the drug to calmodulin [a] (nmol drug bound/mg protein)
Trifluoperazine	18	56 ±6
Chlorpromazine	46	23 ±1
Imipramine	200	6.8±0.6
Diazepam [b]	380	4.7±0.1
Cytochalasin B	>100	
Colchicine	>100	
Lidocaine [b]	> 500	

[a] Values are cited from Ref. 25.

[b] Diazepam and lidocaine were added as an ethanol solution so as to adjust the final concentration of ethanol to 0.5%.

Cytoskeleton-attacking agents, such as cytochalasin B and colchicine, did not affect the vesiculation (Table II). Chlorpromazine induced membrane invagination in erythrocytes [27] and spherical transformation in platelets [28]. As shown in Table II, the effects of 'invaginators' such as lidocaine [29] were also investigated. Lidocaine did not inhibit the exfoliation of acetylcholinesterase activity up to 500 μM.

Little is known about the mechanism of membrane vesiculation. A model for vesiculation has been studied in erythrocytes. Allan et al. [10] proposed that during the process leading to vesiculation of human erythrocytes induced by an ionophore and Ca^{2+}, breakdown of polyphosphoinositide to diacylglycerol may be involved. An alternative possibility was suggested by Ott et al. [11] for phosphatidylcholine-induced shedding of erythrocyte membranes. They proposed that incorporated phosphatidylcholine may move transversely to the inner leaflet, inducing vesiculation through the lateral phase separation of phosphatidylcholine and phosphatidylethanolamine. Recently, Müller

et al. [30] reported that a high concentration of EDTA inhibits vesicle release from ATP-depleted erythrocytes without affecting polyphosphoinositide breakdown and diacylglycerol production. They suggested the involvement of bound cations, possibly of calmodulin, in membrane vesiculation. Our present results, although only the parallelism was indicated between two inhibitions of vesiculation and of calmodulin, are consistent with this suggestion of Müller et al. Furthermore, it could be assumed that the same mechanism underlies the vesiculation of erythrocytes induced by ATP-depletion and phosphatidylcholine-induced shedding of platelet plasma membranes.

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