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EFFECT OF SOME AMPHIPHILIC DRUGS ON THE MEMBRANE MORPHOLOGY AND AGGREGATION OF RABBIT PLATELETS

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Treatment of normal, disc-shaped rabbit platelets with lysophosphatidylcholine and chlorpromazine induced respectively spine formation and spherical transformation. In similar concentration ranges to those in which they induced these morphological changes, the drugs suppressed a series of events triggered by thrombin: pseudopod formation, arachidonate release from the membrane phospholipids, and aggregation. Washing the drug-treated platelets reversed the morphological changes and abolished the inhibitory effect on aggregation. These observations suggest that amphiphilic drugs perturb the plasma membrane structure of platelets, inducing the membrane shape change and inhibiting the stimulus-induced aggregation.

INTRODUCTION:

Platelets play an important role in hemostasis and tend to be aggregated by such stimuli as thrombin and collagen (1), which are known to trigger such membrane phenomena as change in the membrane morphology (i.e., pseudopod formation) (2) and degradation of the membrane phospholipids (i.e., liberation of arachidonic acid from the membrane phospholipids) (3). The close relationship of the membrane morphology and lipid metabolism of platelets with the aggregation phenomenon led us to examine the possible effects of amphiphilic compounds on the platelet membrane, since these compounds are known to induce changes in membrane shape in human erythrocytes through their action on the plasma membrane, possibly on the lipid bilayer (4, 5).

In this work, we studied effects of amphiphilic drugs on the membrane morphology of rabbit platelets and on their phospholipid metabolism and aggregating behavior. Lysophosphatidylcholine and lauric acid were employed

Abbreviations used in this paper: BSA, bovine serum albumin; EDTA, ethylendiaminetetraacetic acid; TBS, 134 mM NaCl-15 mM Tris (pH 7.4)-5 mM glucose.

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as typical drugs which induce membrane crenation in erythrocytes, while chlorpromazine and cepharanthine were used as cationic drugs which induce membrane invagination in erythrocytes.

MATERIALS AND METHODS:

Reagents. Thrombin was purchased from Dade Diagnostic Inc. (Florida, USA), bovine serum albumin (essentially fatty acid-free) (BSA), lysophosphatidylcholine (palmitoyl) and chlorpromazine were from Sigma Chemical Co. (Miss., USA), lauric acid was from Wako Pure Chemical Co. (Osaka, Japan) and [1-^{14}C]-arachidonic acid was from the Radio Chemical Centre (Amersham, England). Cepharanthine (99% purity) was a gift from Kaken Pharmaceutical Co. (Osaka, Japan).

Platelet suspension. Platelets were obtained from rabbit blood anticoagulated with EDTA. Washed platelets suspended in Tyrode solution, pH 7.4, (5×10^5 platelets/μl) were prepared according to the method of Ardlie et al. (6) for the observation of morphology and aggregation. For the experiment on arachidonate release, washed platelets suspended in 134 mM NaCl-15 mM Tris (pH 7.4)-5 mM glucose (TBS) were prepared according to the method of Kannagi et al. (7). All washing and suspending media contained 0.35% BSA.

Platelet aggregation. Platelet aggregation was measured by the turbidimetric method of Born (8). After treatment of platelet suspension (250 μl) with drugs (25 μl) at 37°C for 5 min, platelet aggregation was initiated by adding 25 μl of thrombin (1.2 U/ml) to the platelet suspension at 37°C.

Observation of platelet morphology. Treated platelets were fixed with 2% glutaraldehyde-1% osmic acid, then adsorbed onto coverslips previously coated with poly-L-lysin (9, 10), and processed by serial dehydration in ethanol, critical point drying with liquid CO_2, and coating with gold-palladium. They were examined in a JEOL JSM-35 scanning electron microscope.

Determination of [1-^{14}C]-arachidonate release. Platelets (1×10^6 platelets/μl) in TBS (10 ml) were incubated with 1 μCi of [1-^{14}C]-arachidonic acid at 37°C for 2 hr, treated with 0.2 mM aspirin at 20°C for 30 min, then washed twice with TBS containing 1 mM EDTA (7). The labelled platelets were resuspended in TBS containing 4 mM $CaCl_2$, and 500 μl of the platelet suspension was treated with drugs (50 μl) at 37°C for 5 min, then incubated with 2.5 U/ml of thrombin at 37°C for 1 hr. Lipids extracted according to the method of Folch et al. (11) were separated by two dimensional thin layer chromatography. The developing solvents were chloroform/methanol/25% NH_4OH (90:54:11, by vol.) for the first dimension and chloroform/methanol/acetic acid/distilled water (90:40:12:2, by vol.) for the second. Radioactive spots were localized by autoradiography, and radioactivity in each spot was determined with a Nuclear Cicago Liquid Scintillation Counter (Model Mark III).

RESULTS:

As shown in Fig. 1-A~D, lysophosphatidylcholine transformed the normal platelets in a concentration-dependent manner into spiny discs and spheres with unusual slender filopodia. Similar transformation was observed in the platelets treated with lauric acid (data not shown). Chlorpromazine, on the other hand, induced swelling of the normal disc and sphere formation, also to a degree dependent on the concentration (Fig. 1-E~G). Other cationic amphiphilic drugs, such as cepharanthine and some phenothiazine neuroleptics, induced the same type of membrane shape change (data not shown). When these

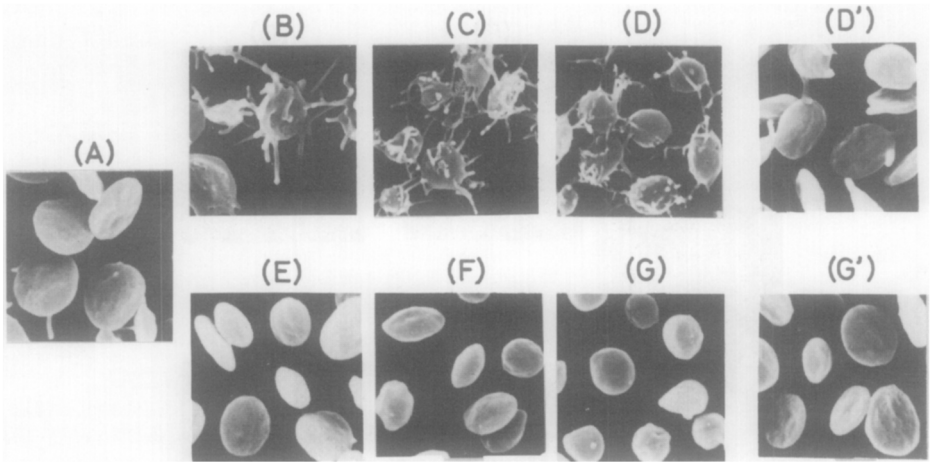

Fig. 1. Membrane shape changes of platelets induced by amphiphilic drugs. Platelets were treated with 0 (A), 75 μM (B), 100 μM (C) and 150 μM (D) of lysophosphatidylcholine, or 50 μM (E), 60 μM (F) and 75 μM (G) of chlorpromazine at 37°C for 5 min. Platelets treated as in D and G were washed three times with Tyrode solution containing 1 mM EDTA and 0.35% BSA to give (D') and (G'), respectively.

drug-treated platelets were washed with BSA-containing Tyrode solution, they recovered an almost normal disc morphology (Fig. 1-D' and G').

Both lysophosphatidylcholine and chlorpromazine inhibited thrombin-induced aggregation of platelets in a concentration-dependent fashion (Fig. 2).

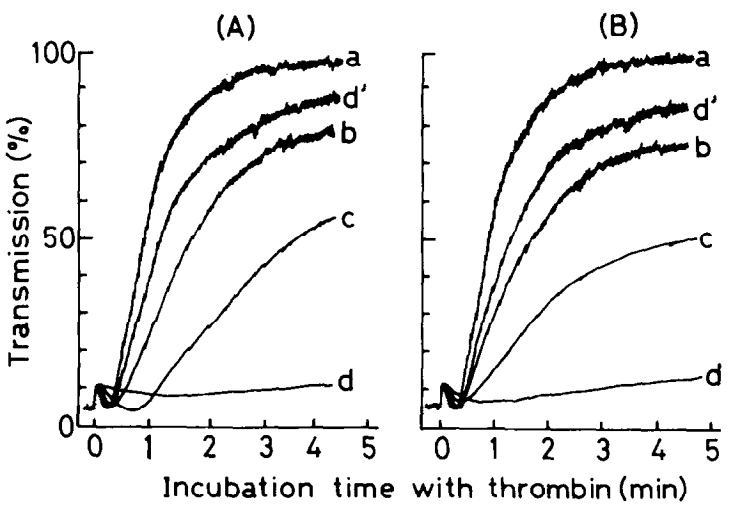

Fig. 2. Inhibitory effect of amphiphilic drugs on the thrombin-induced platelet aggregation. (A) Platelets were treated with 0 (a), 75 μM (b), 100 μM (c) and 150 μM (d) of lysophosphatidylcholine at 37°C for 5 min, then stimulated by adding 0.1 U/ml of thrombin at 37°C under continuous stirring. (B) Platelets treated with 50 μM (b), 60 μM (c) and 75 μM (d) of chlorpromazine at 37°C for 5 min were similarly stimulated with thrombin. Platelets washed after the treatment with 150 μM lysophosphatidylcholine (d' in A) or with 75 μM of chlorpromazine (d' in B) were also stimulated with thrombin.

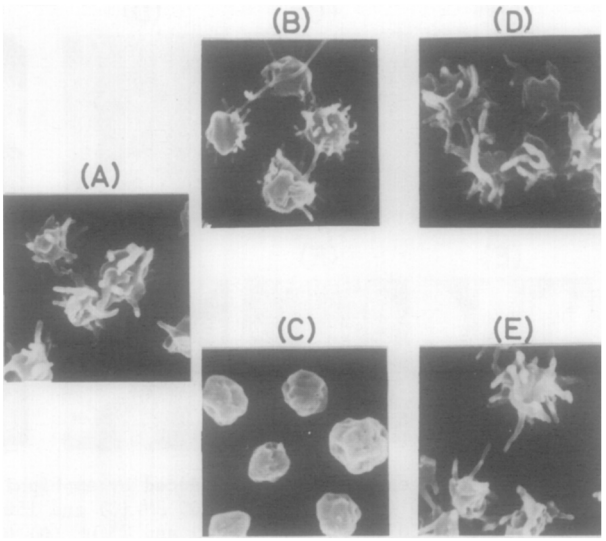

Fig. 3. Effect of amphiphilic drugs on the pseudopod fromation induced by thrombin. Platelets were stimulated with 0.1 U/ml of thrombin at 37°C for 1 min without stirring after incubation without drug (A) or with 150 μM lysophosphatidylcholine (B) and 75 μM chlorpromazine (C) at 37°C for 5 min. The drug-treated platelets (B and C) were washed and similarly stimulated with thrombin (D and E, respectively).

This inhibitory effect was also abolished by washing the drug-treated platelets with BSA-containing Tyrode solution (Fig. 2, curves d').

When normal platelets (Fig. 1-A) were stimulated with thrombin without stirring, native pseudopods were formed without aggregation (Fig. 3-A). However, when lysophosphatidylcholine- and chlorpromazine-treated platelets (corresponding to Fig. 1-D and G, respectively) were stimulated with thrombin, they did not form native pseudopods but retained their abnormal shapes (Fig.

Table I. EFFECT OF AMPHIPHILIC DRUGS ON THROMBIN-INDUCED RELEASE OF [1-^{14}C]-ARACHIDONIC ACID FROM MEMBRANE PHOSPHOLIPIDS OF PLATELETS

treatment		radioactivity in each fraction (%dpm)				
1st	2nd	triglyceride	free fatty acid	phosphatidylcholine	phosphatidylethanolamine	phosphatidylinositol + phosphatidylserine
none	none	2.0	4.9	51.9	27.6	13.6
none	2.5 U/ml thrombin	1.7	38.6	20.4	35.3	4.0
150 μM lysophosphatidylcholine	2.5 U/ml thrombin	2.7	8.5	45.6	33.3	9.9
100 μM chlorpromazine	2.5 U/ml thrombin	2.3	11.2	42.8	34.3	9.4

3-B and C). Native pseudopods were, however, elicited by thrombin-stimulation when the drug-treated platelets were washed with BSA-containing Tyrode solution (Fig. 3-D and E).

As shown in Table I, addition of thrombin to the [1-^{14}C]-arachidonate labelled platelets mobilized about 35% of the radioactive arachidonate, mainly from the phosphatidylcholine fraction and partly from the phsphatidyl-inositol-plus-phosphatidylserine fraction. Pretreatment of the platelets with 150 μM lysophosphatidylcholine or 100 μM chlorpromazine at 37°C for 5 min considerably inhibited the liberation of arachidonic acid from these membrane phsopholipids.

DISCUSSION:

Our results show that amphiphilic drugs induce similar changes in membrane shape in rabbit platelets to those they induce in human erythrocytes, and that they profoundly affect a series of events in thrombin-stimulated platelets: pseudopod formation, liberation of arachidonic acid from the membrane phospholipids, and aggregation. Such "crenator" drugs (so named because they induce crenation in erythrocytes) as lysophosphatidylcholine and lauric acid, induced the formation of numerous abnormal slender philopodia in intact platelets and inhibited the formation of thrombin-induced native pseudopods and aggregation. Such "invaginator" drugs, which induced membrane invagination in erythrocytes, as chlorpromazine and cepharanthine, on the other hand, induced spherical transformation of normal platelets, while also inhibiting the pseudopod formation and aggregation induced by thrombin. It is worth noting that these effects of the drugs on membrane morphology and thrombin-induced aggregation seem to be closely correlated; the membrane-transforming action and the inhibition of aggregation were observed in similar ranges of concentration of the drugs and disappeared upon washing the drug-treated platelets with BSA-containing Tyrode solution, probably due to elimination of the incorporated drug.

At present, the mechanism by which these drugs act on the platelet membrane is obscure. However, we can speculate from our observations that

the amphiphilic drugs may be incorporated into the lipid bilayer of the platelet plasma membrane and perturb its structure. This perturbation may, on one hand, prevent the formation of native pseudopods under the stimulation of thrombin, and, on the other, block the access to the membrane phospholipids of phospholipase A_2, which normally hydrolyzes the phospholipids to liberate arachidonic acid under thrombin stimulation. Prevention of pseudopod formation may prevent the surface interaction between platelelts that is essential for propagation of the activating stimulus from one platelet to another and subsequent aggregation (12). Prevention of liberation of arachidonic acid from membrane phospholipids should prevent activation of the arachidonic acid cascade, which leads to the formation and release of prostaglandin analogs, believed to be essential substances for aggregation. At present, however, it is not clear which of these effects of the drugs is more important in the inhibition of platelet aggregation.

The functions of cytoskeletons in platelets are believed to be responsible for the formation of pseudopods under thrombin stimulation (13). Chlropromazine has been reported to inhibit some of the normal functions of actomyosin and microtubules (14). It is not unlikely, therefore, that amphiphilic drugs act on the cytoskeletal system, though whether the action, if it exists, is direct or indirect, that is, through perturbation of the lipid bilayer of the plasma membrane, remains to be clarified.

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