Acta Medica Okayama

Volume 38, *Issue* 2 1984 *Article* 2

APRIL 1984

Inhibition of platelet aggregation by cepharanthine is accomplished during the early, membrane-related activation process.

Sadahiro Watanabe*

*Okayama University,

Inhibition of platelet aggregation by cepharanthine is accomplished during the early, membrane-related activation process.*

Sadahiro Watanabe

Abstract

Cepharanthine, a biscoclaurine alkaloids which interact with biomembranes, has been found to inhibit platelet aggregation. The effects of this drug on morphological and physiochemical phenomena following collagen-induced platelet stimulation were investigated. In the presence of cepharanthine, stimulated platelets became spherical, but did not form pseudopoda , nor did they become aggregated. Physiochemical reactions such as accelerated oxygen consumption, release of membrane-bound Ca2+, release of Ca2+ into the extracellular medium and deporalization of the membrane potential were all inhibited by cepharanthine. Using D,L-dipalmitoyl phosphatidylcholine liposomes as the substrate, cepharanthine was shown to inhibit phospholipase A2 activity. These results suggest that the changes in the membrane following the interaction of collagen with its receptor are important for platelet activation. Cepharanthine may inhibits these membrane state changes, thus blocking all subsequent reactions.

KEYWORDS: platelet aggregation, cepharanthine, electron microscopy, phospholipase A_2, liposome

---

*PMID: 6731025 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 38, (2), 101-115 (1984)

INHIBITION OF PLATELET AGGREGATION BY CEPHARANTHINE IS ACCOMPLISHED DURING THE EARLY, MEMBRANE-RELATED ACTIVATION PROCESS

Sadahiro WATANABE

Department of Anatomy, Okayama University Medical School, Okayama 700, Japan

Received November 24, 1983

Abstract. Cepharanthine, a biscoclaurine alkaloids which interact with biomembranes, has been found to inhibit platelet aggregation (1). The effects of this drug on morphological and physiochemical phenomena following collagen-induced platelet stimulation were investigated. In the presence of cepharanthine, stimulated platelets became spherical, but did not form pseudopoda, nor did they become aggregated. Physiochemical reactions such as accelerated oxygen consumption, release of membrane-bound Ca^{2+}, release of Ca^{2+} into the extracellular medium and deporalization of the membrane potential were all inhibited by cepharanthine. Using D,L-dipalmitoyl phosphatidylcholine liposomes as the substrate, cepharanthine was shown to inhibit phospholipase A_2 activity. These results suggest that the changes in the membrane following the interaction of collagen with its receptor are important for platelet activation. Cepharanthine may inhibits these membrane state changes, thus blocking all subsequent reactions.

Key words : platelet aggregation, cepharanthine, electron microscopy, phospholipase A_2, liposome.

Many biological responses are influenced by extracellular signals which must first be recognized by cell surface receptors. Transmembrane control, therefore, depends on the physiochemical state of the biomembrane. Recently, platelets have been recognized as being useful for studying transmembrane control system because of their remarkable responses to several molecules such as collagen, thrombin and ADP which produce platelet aggregation *in vitro* (2, 3). These stimulators bind to the platelet surface membrane resulting in induction of intracellular activation. Cepharanthine, a biscoclaurine alkaloids, inhibits platelet aggregation in a dose-dependent manner (1). This potent drug stabilizes biomembranes. Cepharathine inhibits K^+ release from red blood cells (4) and histamine release from must cells (5) and decreases membrane fluidity as observed by inhibition of concanavalin A cap formation (6, 7).

Platelet aggregation is accompanied by several metabolic changes such as accelerated O_2 consumption (8), decrease in membrane bound Ca^{2+} (9), Ca^{2+} release into the extracellular medium (10), membrane potential changes (11) and accelerated phospholipid turnover (12). At present, it is not clear how these

biological changes correlate, and which responses are key to platelet activation. In addition, it is not established how changes in platelet morphology, which are thought to represent cell activation, relate to these metabolic changes. In this study, the inhibitory effect of cepharanthine on platelet aggregation was studied in relation to these changes in morphology and metabolism.

MATERIALS AND METHODS

Platelets. After adding acid citrate dextrose (ACD), whole blood was centrifuged at 375 × g for 10 min at 22 ℃ to obtain platelet-rich plasma (PRP). Further centrifugation of PRP at 3000 × g for 15 min at 22 ℃, resulted in a condensed platelet fraction (PC). Unless otherwise stated in the text, PC was suspended in Ca^{2+}-free Krebs Ringer phosphate solution (KRP) (154 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 10 mM Na-phosphate buffer, pH 7.4) at 37 ℃, at a concentration of approximately 2-4 × 10^5 cells/μl.

Platelet aggregation and electron microscopy. PRP preparations were used for platelet aggregation and morphology studies. Collagen-induced platelet aggregation was monitored with a spectrophotometer (Shimazu UV-200, Kyoto) at 610 nm, equipped with a temperature control unit (37 ℃) and magnetic stirrer. Aggregated platelets were studied by electron microscopy. Two ml of 0.5 % glutaraldehyde (GA) in two-fold concentrated KRP was added to 2 ml of PRP, and cells were slightly fixed for 10 to 15 min at 37 ℃. These cells were further fixed with 2.5 % GA in KRP at 37 ℃ for 1 h. After washing the cells in KRP at 4 ℃ overnight, post-fixation with 1 % OsO_4 in H_2O was performed for 1 h at room temperature. Following dehydration in a graded ethanol series, samples were embedded in Spurr's low viscosity embedding medium (Nakarai Chemical Ltd, Kyoto) (13). Thin sections were obtained with a Sorvall Ultramicrotome (MT-5000, Connecticut), and were stained with uranyl acetate and lead citrate. Photos were taken under a JEOL 100-CX (Tokyo) electron microscope operated at 80 kV.

Changes in membrane potential. Changes in the platelet membrane potential during aggregation were measured with a tetraphenyl phosphonium (TPP) electrode prepared in this labolatory according to the method of Okimasu *et al.* (14). In order to incorporate the TPP into platelets, cells were incubated in KRP containing 10^{-5}M TPP at 37 ℃ with moderate stirring. After the incorporation had equilibrated (15 to 30 min), aggregation was induced. Changes in the TPP concentration in the medium was monitored by the electrode.

Ca^{2+} ion movement. Release of membrane-bound Ca^{2+} into the cytoplasm was measured by changes in fluorescence intensity of chlortetracycline (CTC) by the method of Feinstein (9). PC (1.5 ml) was incubated with 10 μM CTC in an Eppendorf microcentrifugal tube for 30 min at room temperature. After centrifugation at 1500 × g for 30-40 sec, the supernatant was removed and cells were suspended in KRP to 2-4 × 10^5 cells/μl. Aggregation was induced after 30 min. Changes in fluorescence intensity were monitored with a Shimazu fluorospectrophotometer (RF-50, Kyoto) at a wavelength of 540 nm (excitation at 390 nm), equipped with a stirrer and temperature control system (37 ℃).

Ca^{2+} release from the platelets to the extracellular medium was measured by a Ca^{2+} electrode (Orion Reserch, model 93-20, Cambridge).

Oxygen consumption. Oxygen consumption accompanying platelet aggregation was measured by an oxygen electrode (Yellow Spring Instrument, 4004 Clark oxygen probe, Ohio) in a semi-closed cuvette.

Preparation of liposomes. Unilamellar vesicles were prepared from D,L-dipalmitoyl phospha-

tidylcholine (DPPC) by the method of Huang (15). DPPC was dissolved in chloroform-methanol (2 : 1) to a concentration of 22 mg/ml. One ml of the solution was evaporated in a concentrator (Taiyo TC-8, Tokyo). After addition of 2 ml of 0.1 M NaCl, 20 mM phosphate buffer (pH 6.8), liposomes were formed by agitation in a vortex mixer for 10 min at 50 ℃, followed by sonication with a Bransonic sonifier (Type 185, Danbury) for 1 h at 50 ℃. If carboxyfluorescein (CF)-containing liposomes were needed, CF (0.1 M) was added before agitation. In order to obtain uniform unilamellar liposomes, samples were passed through a Sepharose 4B (Pharmacia, Uppsala) column (1.5 × 15 cm), with elution at 4 ℃ with 0.1 M NaCl-20 mM phosphate buffer, pH 6.8.

Phase transition release (PTR). PTR of CF was performed by the modified method (16) of Weinstein *et al.* (17). Changes in fluorescence intensity following the release of CF from liposomes were monitored using a Shimazu fluorospectrophotometer (Type RF-50) at 515 nm (excitation at 470 nm). CF-containing unilamellar liposomes were suspended in an ice-cold cuvette and the cuvette was transferred to a thermostatically controlled cuvette holder to elevate the temperature. The temperature was controlled by a circulation system, and the sample temperature was measured by a digital temperature thermister. The suspension was agitated with a magnetic stirrer. Total CF intensity was determined by the addition of 0.0025 % Triton X-100. Phospholipase A_2, $CaCl_2$ (1 mM) and cepharanthine were added before elevating the temperature.

Phospholipase A_2 (PLA_2) activity. In order to measure PLA_2 activity, DPPC liposomes (750 μM) were incubated with PLA_2 (10 μM) in 0.15 M NaCl, 1 mM Tris-HCl (pH 8.0) with 1 mM $CaCl_2$. PH changes resulting from the formation of fatty acids were monitored with a pH electrode (Horiba Seisakusho Co. Ltd., Kyoto) at 38 ℃. In addition, PLA_2 activity was assayed by lysophosphatidylcholine formation. After incubation of DPPC liposomes (375 μM) with PLA_2 (50 μg/ml) at 10 ℃ for one min, lysophosphatidylcholine and DPPC were separated by thin layer chromatography, and the amount of lysophosphatidylcholine formed was determined by the phosphorus content using the method of Lowry *et al.* (18).

Reagents. Cepharanthine was obtained from Kaken Pharmaceutical Co., Ltd. (Tokyo) and dissolved in ethanol. Collagen (Sigma Type I, Saint Louis) was suspended in 0.15 M NaCl. D,L-dipalmitoyl phosphatidylcholine was purchased from Sigma, and phospholipase A_2, from porcine pancreas, was from Boehringer (Mannheim). Carboxyfluorescein from Eastman Kodak Co. (Rochester) was purified by the method of Ralston *et al.* (19). Other chemicals used were of analytical grade.

RESULTS

Structural changes during collagen-induced platelet aggregation and its inhibition by cepharanthine. Decrease in absorbance at 610 nm (A_{610}), which indicates cell aggregation (20, 21), was noted after exposure of PRP to collagen in the absence of cepharanthine (Fig. 1). In the presence of cepharanthine (25 μM), there was no decrease in optical density. As a control, ethanol without cepharanthine was added. The results were similar to those when no cepharanthine was added.

At various times during the collagen-induced aggregation, samples were prepared for transmission electron microscopy (Fig. 2 and 3). Non-activated, normal platelets demonstrated multiform sections which were circular or spindular depending on the cutting angle (Fig. 2a). An initial slight increase in A_{610}, 1 min after

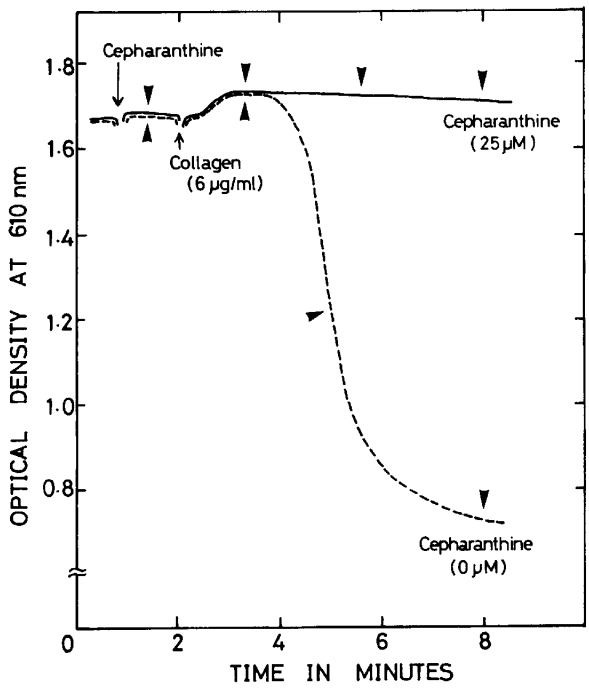

Fig. 1. Inhibition of platelet aggregation by cepharanthine. Time course of collagen-induced platelet aggregation was measured by spectrophotometry. Optical density (610 nm) decreased with aggregation. Samples for electron microscopy were fixed at various times (arrow heads). Ethanol was added instead of cepharanthine in the control experiment.

collagen stimulation, was observed both in the presence and absence of cepharanthine (Fig. 1), and was accompanied by a morphological change in platelets from a discoidal form to either a spherical form or a form with pseudopoda. Platelets then became irregular in shape, and fine pseudopoda were observed (Fig. 2b). The number of discoidal platelets decreased as spindular sections were no longer observed. The decrease in A_{610} was mirrored by cell aggregation. Three min after the addition of collagen, many pseudopoda were entangled and small aggregates formed (Fig. 2c). By about 6 min after stimulation, the aggregation reached maximum levels. Cell aggregates were large, and platelets were interdigitated (Fig. 2b).

No morphological change in platelets was observed by adding cepharanthine alone (Fig. 2a and 3a). After collagen stimulation, however, cepharanthine prevented the morphological changes in platelets. One min after stimulation, platelets showed no remarkable change in shape (Fig. 3b). Three min after stimulation, spindular form sections were decreased indicating cell spherization (Fig. 3c), but neither aggregate nor pseudopoda formation was observed. These forms were also observed at 6 min after stimulation (Fig. 3d).

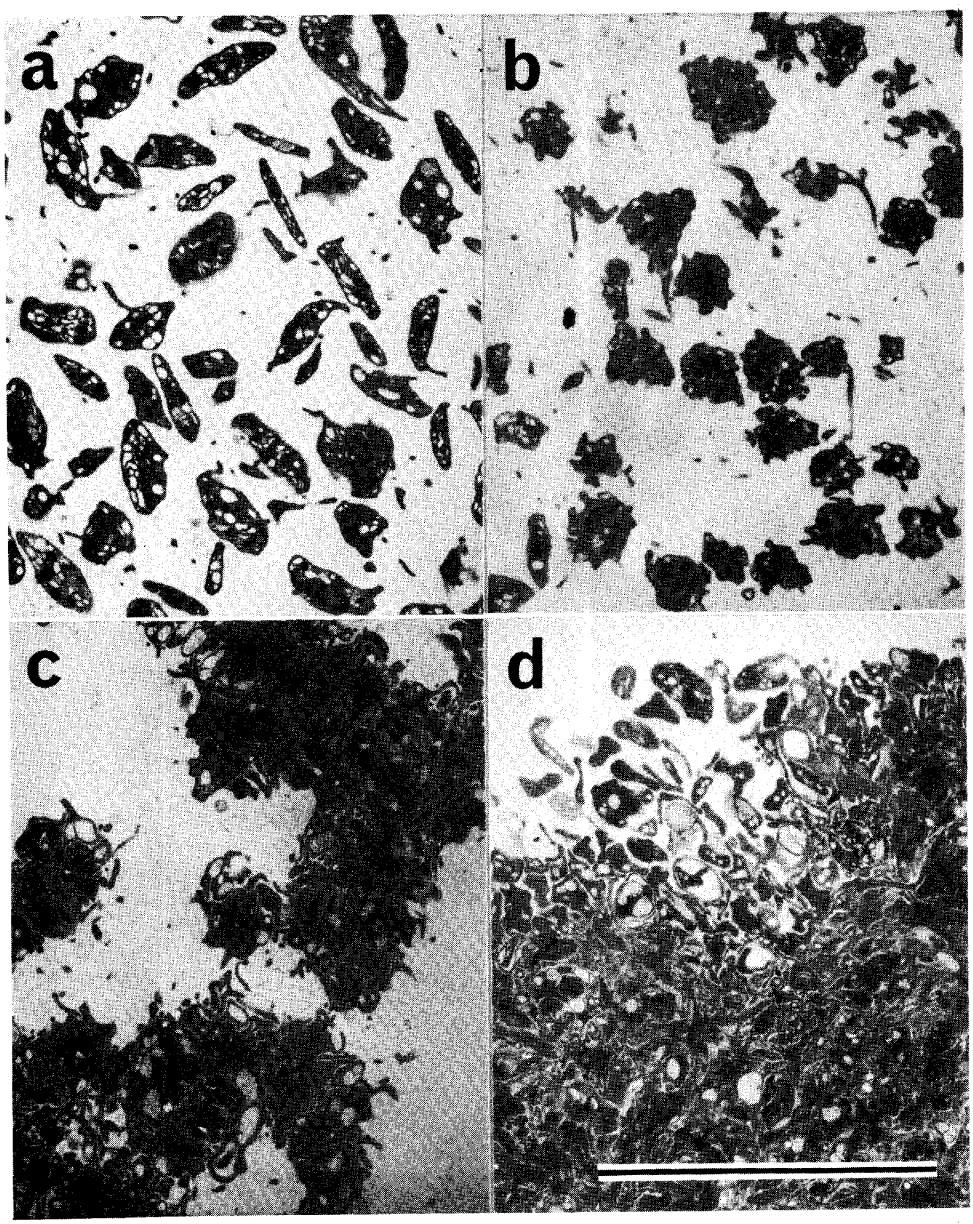

Fig. 2. Electron microscopy of collagen-induced platelet aggregation in the absence of cepharanthine. (a) Platelets before collagen stimulation, predominantly discoid. (b) Platelets 1 min after stimulation, irregular margins and fine pseudopoda are seen. (c) Platelets during aggregation 3 min after stimulation ; note entangled pseudopoda. (d) Large aggregates formed 6 min after stimulation. Bar : 10μm.

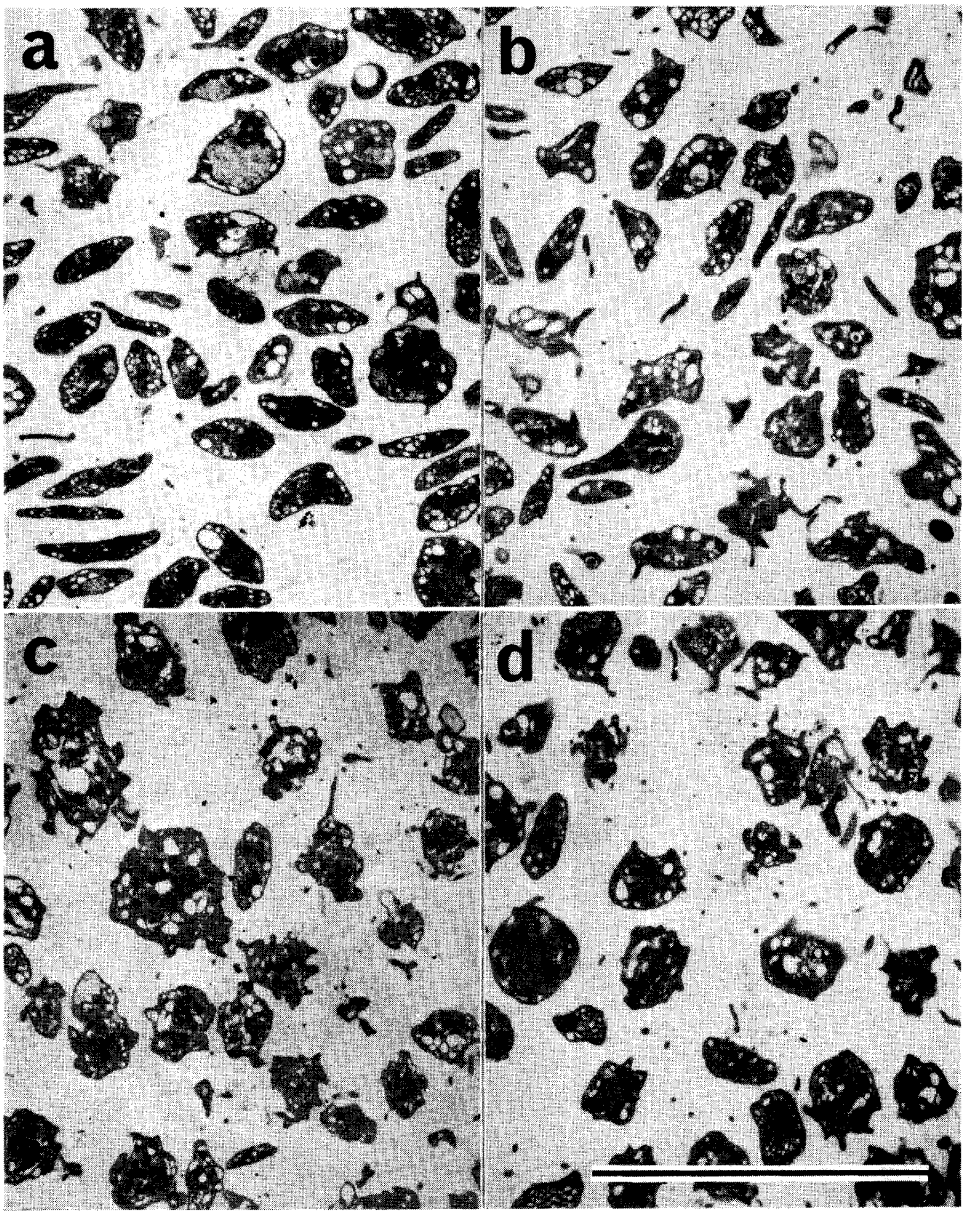

Fig. 3. Electron microscopy of collagen-induced platelet aggregation in the presence of cepharanthine (25 μM). (a) Platelets before collagen stimulation. (b) Platelets 1 min after stimulation, no obvious morphological changes. (c) Platelets 3 min after collagen stimulation, some irregular forms are present. (d) Platelets 6 min after stimulation, similar in shape to those in Fig. 3c. Bar : 10 μm.

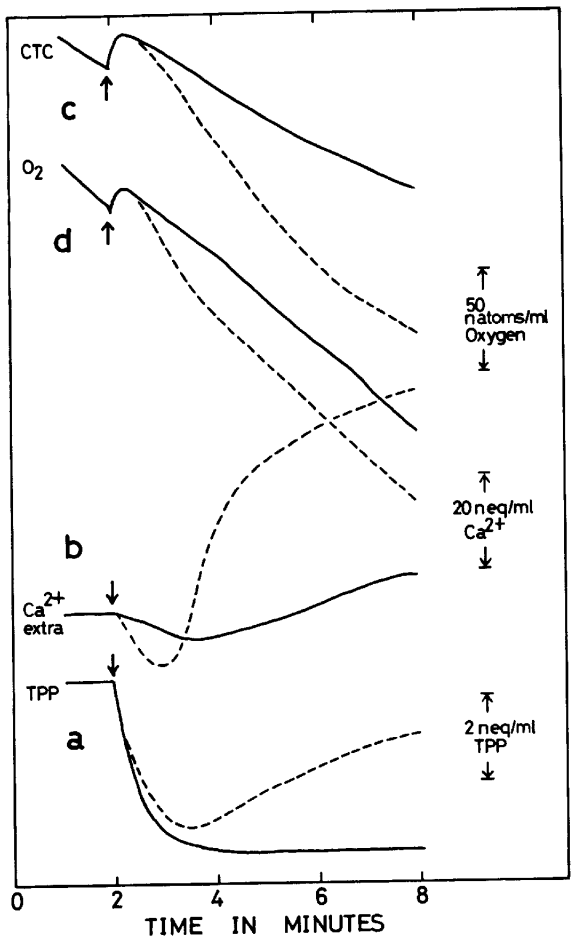

Fig. 4. Effect of cepharanthine on four different responses in stimulated platelets. Arrows mark the addition of collagen. Dotted line (----) : in the absence of cepharanthine. Solid line (—) : in the presence of cepharanthine. (a) Changes in membrane potential were monitored by changes in the TPP concentration in the medium. (b) The release of intracellular Ca^{2+} of platelets to extracellular medium was measured by a Ca^{2+} electrode. (c) Membrane-bound Ca^{2+} was measured by the change of fluorescence of CTC. (d) Oxygen consumption measured by an O_2 electrode.

Changes in various parameters of platelets stimulated by collagen. Several physiological parameters were measured simultaneously after induction of aggregation by collagen (Fig. 4). The measurements included membrane potential, extracellular and membrane-bound calcium contents, and oxygen consumption.

Membrane potential changes in platelets were measured with a tetraphenyl phosphonium (TPP) electrode. TPP molecules diffuse electrophoretically across biological membranes, and the binding of TPP on the membrane surface is negligibly small for calculation of the membrane potential (22). Therefore, change

in TPP concentration in the incubation medium reflect membrane potential changes. During the first minute after addition of collagen, the TPP concentration fell as the membrane hyperpolarized, *i.e.*, the TPP in the medium decreased (Fig. 4a). This deflection was artifactual, due to the suspension medium (0.15 M NaCl) of collagen. Subsequently, depolarization of the membrane potential, *i.e.*, an increase in TPP in the medium, was observed only in the absence of cepharanthine. This depolarization was not induced by suspension medium without collagen.

The release of intracellular Ca^{2+} to the extracellular medium was measured with a Ca^{2+} electrode (Fig. 4b). By addition of cepharanthine, this release was greatly reduced. Release of membrane-bound Ca^{2+} was monitored by fluorescence from CTC (9). CTC molecules chelate Ca^{2+} ions and fluoresce in hydrophobic environments such as a phospholipid bilayer (23). Rapid decrease in fluorescence of CTC by stimulation with collagen indicated that membrane-bound Ca^{2+} moved to the cytoplasm (Fig. 4c). In the presence of cepharanthine, this Ca^{2+} ion movement was inhibited.

Non-stimulated platelets constantly take up oxygen, this being sensitive to mitochondrial respiration inhibitors. O_2 uptake increased rapidly for a short time

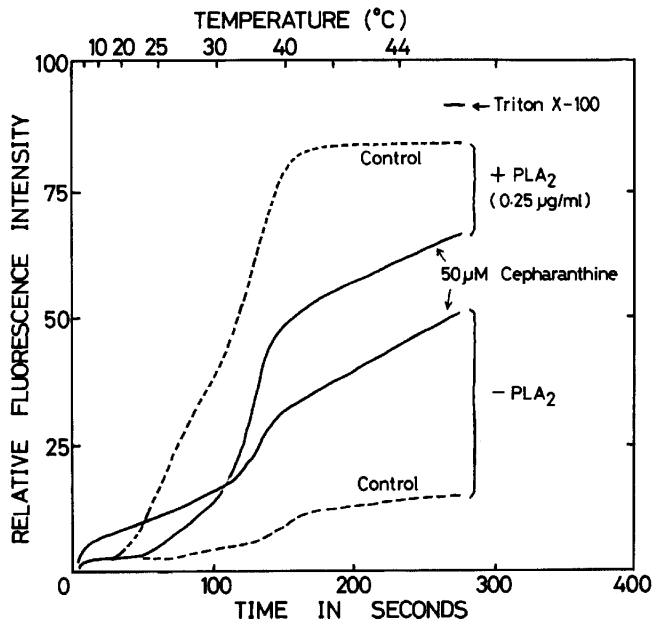

Fig. 5. The effect of cepharanthine on phase transition release with or without PLA_2. Liposome containing fluorescent material (CF) was incubated in ice-cold buffer and warmed to 44 ℃. The temperature in the cuvette reached 30 ℃ in 100 seconds and 44 ℃ within 200-250 seconds. Addition of Triton X-100 (0.0025 %) resulted in maximum CF release.

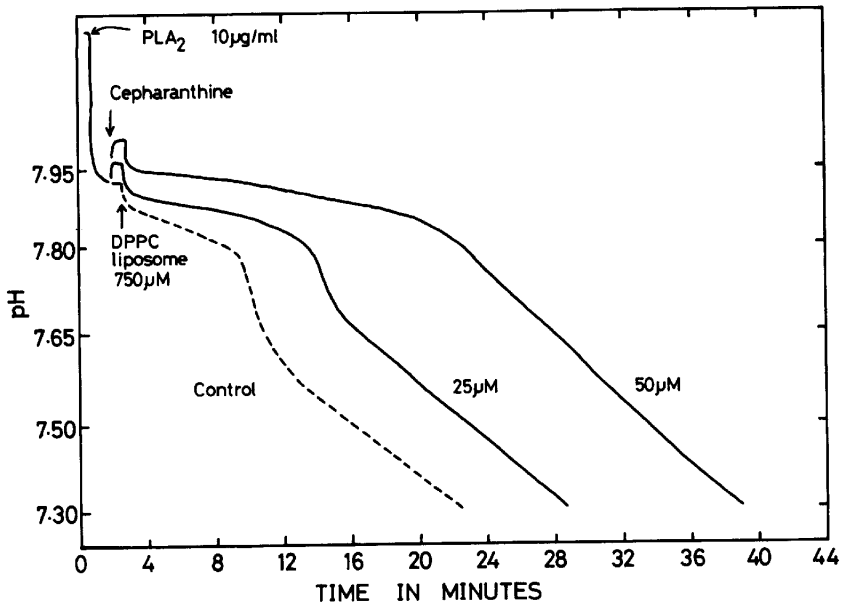

Fig. 6. The effect of cepharanthine on PLA_2 activity observed by the pH change in DPPC liposome suspension. The pH change reflects fatty acid formation by PLA_2. Performed at 38 ℃, the phase transition temperature (Tc).

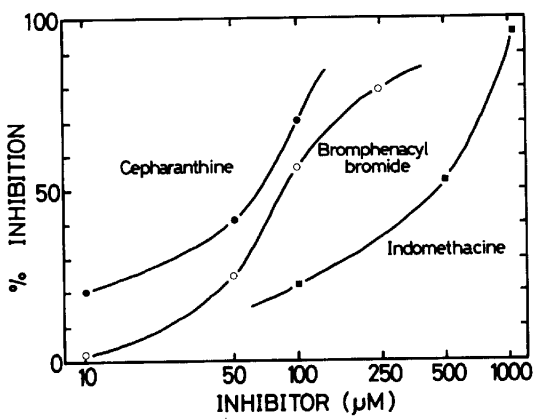

Fig. 7. Inhibitory effects of cepharanthine, bromophenacyl bromide and indomethacin on lysophosphatidylcholine formation from PLA_2-treated DPPC liposomes. Inhibition is expressed as percent inhibition.

period following addition of collagen, and then returned to approximately the same levels of pre-stimulation (Fig. 4d). This transient increase in O_2 uptake was cyanide insensitive and probably due to the oxygenation of arachidonic acid,

the "O_2 burst" (10, 24). The O_2 burst started about 1 min after addition of collagen, which coincided with the transitional increase in A_{610}. Addition of cepharanthine eliminated the O_2 burst, but the rate of O_2 uptake for mitochondrial respiration was not affected.

The effect of cepharanthine on PLA_2 activity as determined by PTR, pH change, and thin layer chromatography. One of the most important changes coupled with the initial step of platelet aggregation is the activation of phospholipase A_2 activity, the starting point of the arachidonic acid cascade (25). From the above experimental data, it was postulated that cepharanthine regulated the membrane-bound enzyme activity. Therefore, the effect of cepharanthine on PLA_2 activity was investigated using artificial phospholipid liposomes.

DPPC liposomes containing carboxyfluorescein (CF) are useful for detecting PLA_2 activity, since CF release from such liposomes increases in the presence of PLA_2. In a study using a phase transition release technique, a small amount of CF was released below the phase transition temperature (Tc), which was 38 ℃ (Fig. 5). Cepharanthine caused membrane perturbations and resulted in additional CF release. In the presence of PLA_2 (+ PLA_2), however, cepharanthine reduced the rate of PLA_2-induced release from liposomes. This inhibitory effect was dose dependent (data not shown).

PLA_2 activity resulted in the formation of lysolecithin and free fatty acids which lower the pH of the medium. The inhibitory action of cepharanthine on PLA_2 activity was further confirmed by measuring pH change in the liposome suspension (Fig. 6). At Tc (38 ℃), there was a lag time for PLA_2 activity to be expressed as a change in pH. This lag time was prolonged by the addition of cepharanthine. The lag time increased in a dose dependent manner. However, the rate of pH decrease, once initiated, was independent of cepharanthine.

Formation of lysolecithin by PLA_2 was studied by thin layer chromatography (Fig. 7). Inhibition of lysolecithin formation by cepharanthine, bromophenacyl bromide (BPB), a well known inhibitor of PLA_2 (26), and indomethacin, a cyclooxygenase inhibitor, were compared. Cepharanthine exhibited stronger inhibitory action than BPB.

DISCUSSION

It is believed that cepharanthine is incorporated into the phospholipid bilayer, lowering the fluidity of and stabilizing the membrane phospholipids (4, 5, 6, 7). In this study, membrane phenomena following platelet stimulation with collagen were investigated in the presence of cepharanthine. Understanding the effect of this drug is critical because the first step in platelet activation is believed to occur in the cell membrane.

Platelets change their shape with stimulation (27). In resting platelets, the characteristic discoid shape is maintained by microtubule coils distributed just beneath the plasma membrane as a circular ring (28). Platelet spherization and

pseudopod protrusions are observed shortly after stimulation. Since the pseudopoda become entangled to form aggregates, pseudopod formation is thought to be important for collagen-induced platelet aggregation. Cepharanthine inhibited pseudopod formation but not spherization. The mechanism of spherization is unclear. Formation of pseudopoda, however, probably depends on actin polymerization, because platelet monomeric actin content decreases and actin polymerization occurs upon stimulation (29, 30). Several other cytoskeletal, actin binding proteins, such as myosin and a 31 K protein, are thought to participate in this change in cyto-architecture (31-34). A recent concept for the pathway of intracellular cytoskeletal alteration involves phosphorylation of the myosin light chain (MLC) and myosin light chain kinase (MLCK). The latter enzyme is phosphorylated by C-kinase which requires Ca^{2+}, membrane phospholipids and diacylglyceride for its activation (35-38). Therefore, it is possible that cepharanthine inhibited C-kinase by suppressing the membrane-bound Ca^{2+} release and, indirectly, changes in cytoskeletal architecture. According to an alternative hypothesis, thromboxane A_2 (TxA_2), a product of arachidonic acid (AA), acts as a Ca^{2+} ionophore, supplying Ca^{2+} ions to several reactions, including the actin-myosin interaction (39). TxA_2 production is dependent on PLA_2, a membrane-bound enzyme. In either case, the inhibitory effect of cepharanthine on platelet cytoskeletal changes may be mediated through membrane stabilization.

Rapid oxygen consumption or the "O^2 burst" observed after platelet activation is cyanide insensitive and considered to be related mainly to oxygenation of arachidonic acid and secondarily to mitochondrial respiration (10, 24). This O_2 burst must be preceded by release of AA by the activation of PLA_2 on membrane phospholipid. As cepharanthine does not inhibit mitochondrial O_2 consumption (40, 41), inhibition of the O_2 burst can be explained by the inhibition of PLA_2.

Increased cytoplasmic Ca^{2+} is derived from the membrane after stimulation with collagen. This increase in Ca^{2+} concentration is involved in early platelet activation (9, 42). The decrease in CTC fluorescence intensity indicated the release of the membrane-bound Ca^{2+} to the extracellular fluid. The mechanism of membrane-bound Ca^{2+} release is not known, but has an important role in the various metabolic changes in platelets mentioned above. Calcium ions were released into the extracellular medium from dense bodies as part of the aggregation response (43). The secretory process is accompanied by centralization of intracellular granules, this being closely correlated to activation of cytoplasmic contractile or cytoskeletal proteins (44).

Changes in the membrane potential reflect changes in membrane permeability to Na^+ or K^+ ions (11), and have an important role in the early events of cell activation. In the present study, TPP, a lipophilic cation, was transported into cells in response to membrane potential changes. Collagen-induced depolarization of the platelet membrane may be closely related to the increase in membrane permeability. If so, cepharanthine may inhibit the depolarization by modification

of the cell membrane.

An early event in platelet stimulation is AA formation. Two different pathways for the release of AA have been proposed. One is via PLA_2 which hydrolysis AA from the 2 position of phospholipids (25) ; the alternative involves phospholipase C (PLC), which forms 1, 2-diacylglycerol from phosphatidylinositol, followed by liberation of AA by diacylglycerol lipase (45). Kanaho *et al.* (46) have suggested that cepharanthine inhibits PLA_2. The present study has shown the inhibition of PLA_2 by cepharanthine using a number of approaches. Activation of PLA_2 involves several factors, the most important of which is Ca^{2+} ions. Additionally contact of substrate and enzyme probably depends on the physical state of the membrane. It is probably that cepharanthine alters the membrane state disadvantageously for PLA_2. As yet, however, this remains speculative.

Many of the metabolic changes in activated platelets may be secondary to changes in the physiochemical state of the platelet membrane. The inhibitory action of cepharanthine can be explained by membrane modification with this drug which eliminate the membrane changes required for platelet activation, suggesting that the initial step for platelet activation is initiated in the membrane. Recently, as one of the other problem concerning platelet aggregation, it was reported that the appearance of fibrinogen receptors on the platelet surface was controlled by stimulation with ADP and thrombin (47, 48). It will be necessary to study further the effect of cepharanthine on this receptor mobility.

Acknowledgements. The author gratefully acknowledges the direction of Prof. Nagayasu Otsuka, Department of Anatomy, Okayama University Medical School, and Prof. Kozo Utsumi, Department of Medical Biology, Kochi Medical School. I am also grateful to Dr. Junzo Sasaki, Department of Anatomy, Okayama University Medical School, for his critical reading of the manuscript. Several important suggestions and technical advice were given kindly by Dr. Masanobu Miyahara and Mr. Eiji Okimasu, Department of Medical Biology, Kochi Medical School, and Mr. Noriyuki Shiraishi, Department of Radiation Medicine, Okayama University Medical School. I am also indebted to Mrs. Yasuko M. Morimoto, Japan Red Cross Blood Center, Kochi Branch, for her continuous supply of platelets, and for the Kaken Pharmaceutical Company of Tokyo for the donation of cepharanthine.

REFERENCES

1. Watanabe, S., Morimoto, Y.M., Shiraishi, N., Sano, A. and Utsumi, K.: The inhibition of platelet aggregation by biscoclaurine alkaloids. *Cell Struct. Funct.* **6** 263-267, 1981.
2. Gaarder, A., Jonsen, J. and Laland, S.: Adenosine diphosphate in red blood cells as a factor in the adhesiveness of human blood platelets. *Nature* **192**, 531-532, 1961.
3. Mustard, J.F. and Packham, M.A.: Factors influencing platelet function; adehesion, release and aggregation. *Pharmacol. Rev.* **22**, 97-187, 1970.
4. Utsumi, K., Miyahara, M., Inoue, M., Mori, M., Sugiyama, K. and Sasaki, J. : Inhibition by cepharanthine of red blood cell potassium release by lead acetate and lysolecithin. *Cell Struct. Funct.* **1**, 133-136, 1976.
5. Sugiyama, K., Sasaki, J., Utsumi, K. and Miyahara, M. : Inhibition by cepharanthine of histamine release from rat peritoneal mast cells. *Allergy* **25**, 685-691, 1976 (in Japanese).

6. Sugiyama, K. and Utsumi, K.: FITC-Con A induced cap formation of Ehrlich ascites tumor cells and its inhibition. *Igaku no Ayumi* **102**, 703-705, 1976 (in Japanese).
7. Utsumi, K., Miyahara, M., Sugiyama, K. and Sasaki, J.: Effect of biscoclaurine alkaloids on the cell membrane related membrane fluidity. *Acta Histochem. Cytochem.* **9**, 59-68, 1976.
8. Bressler, N.M., Broekman, M.J. and Marcus, A.J.: Concurrent studies of oxygen consumption and aggregation in stimulated human platelets. *Blood* **53**, 167-178, 1979.
9. Feinstein, M.B.: Release of intracellular membrane-bound calcium precedes the onset of stimulus-induced exocytosis in platelets. *Biochim. Biophys. Res. Commun.* **93**, 593-600, 1980.
10. Akkerman, J.W.N. and Holmsen, H.: Interrelationship among platelet responses: Studies on the burst in proton liberation, lactate production, and oxygen uptake during platelet aggregation and Ca^{2+} secretion. *Blood* **57**, 956-966, 1981.
11. Horne, W.C. and Simons, E.R.: Probes of transmembrane potentials in platelets: Changes in cyanine dye fluorescence in response to aggregation stimuli. *Blood* **51**, 741-749, 1978.
12. Bills, T.K., Smith, J.B. and Silver, M.J.: Metabolism of (1-^{14}C) arachidonic acid by human platelets. *Biochim. Biophys. Acta* **424**, 303-314, 1976.
13. Spurr, A.R.: A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31-43, 1969.
14. Okimasu, E., Kuroki, M., Utsumi, T., Kobayashi, S. and Utsumi, K.: A criticism of the measurement of membrane potential change by means of lipophilic ions. *Med. Res. Photosensitive Drug* **88**, 48-56, 1981 (in Japanese).
15. Huang, C.: Studies of phosphatidylcholine vesicles. Formation and characteristics. *Biochem.* **8**, 344-352, 1969.
16. Okimasu, E., Shiraishi, N., Kobayashi, S., Morimoto, Y.M., Miyahara, M. and Utsumi, K.: Permeability changes of phospholipid liposomes caused by pancreatic phospholipase A_2: analysis by means of phase transition release. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **145**, 82-86, 1982.
17. Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E. and Blumenthal, R.: Phase transition release, a new approach to the interaction of proteins with lipid vesicles. *Biochim. Biophys. Acta* **647**, 270-289, 1981.
18. Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L. and Farr, A.L.: The quantitative histochemistry of brain. I. Chemical method. *J. Biol. Chem.* **207**, 1-17, 1954.
19. Ralston, E., Hjelemeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R.: Carboxyfluorescein as a probe for liposome-cell interactions. Effect of impurities and purification of the dye. *Biochim. Biophys. Acta* **469**, 133-137, 1981.
20. Born, G.V.R.: Aggregation of blood platelets by adenosine phosphate and its reversal. *Nature* **194**, 927-929, 1962.
21. O'Brien, J.R.: Platelet aggregation. II. Some results from a new method of study. *J. Clin. Pathol.* **15**, 452-455, 1962.
22. Muratsugu, M., Kamo, N., Kobatake, Y. and Suda, M.: Monitoring of the membrane potentials in *E. coli.* and in mitochondria by use of a selective-electrode for tetraphenyl phosphonium ion (TPP). *Membrane* **4**, 323-330, 1978 (in Japanese).
23. Caswell, A.H. and Hutchinson, J.D.: Visualization of membrane bound cations by a fluorescent technique. *Biochim. Biophys. Res. Commun.* **42**, 43-49, 1971.
24. Pickett, W.C. and Cohen, P.: Mechanism of the thrombin-mediated burst in oxygen consumption by human platelets. *J. Biol. Chem.* **251**, 2536-2538, 1976.
25. Billah, M.M., Lapetina, E.G. and Cuatrecasas, P.: Phospholipase A_2 and phospholipase C activities of platelets. Differential substrate specificity, Ca^{2+} requirement, pH dependence and

cellular localization. *J. Biol. Chem.* **255**, 10227-10231, 1980.

26. Roberts, M.F., Deems, R.A., Mimcey, T.C. and Deenis, E.A.: Chemical modification of the histidine residue in phospholipase A_2 (*Naja naja*). A case of half-site reactivity. *J. Biol. Chem.* **252**, 2405-2411, 1977.
27. Nachmias, V.T.: Cytoskeleton of human platelets at rest and after spreading. *J. Cell Biol.* **86**, 795-802, 1980.
28. White, J.G.: Fine structural alterations induced in platelets by adenosine diphosphate. *Blood* **31**, 604-622, 1968.
29. Carroll, R.C., Butler, R.G., Morris, P.A. and Gerrard, J.M.: Separable assembly of platelet pseudopodal and contractile cytoskeletons. *Cell* **30**, 385-393, 1982.
30. Jennings, K.L., Fox, J.E.B., Edwards, H.H. and Phillips, D.R.: Changes in the cytoskeletal structure of human platelets following thrombin activation. *J. Biol. Chem.* **256**, 6927-6932, 1981.
31. George, J.N., Lyons, R.M. and Morgan, R.K.: Membrane changes associated with platelet activation. Exposure of actin on the platelet surface after thrombin-induced secretion. *J. Clin. Invest.* **66**, 1-9, 1980.
32. Daniel, J.L., Molish, I.R. and Holmsen, H.: Myosin phosphorylation in intact platelets. *J. Biol. Chem.* **256**, 7510-7514, 1981.
33. Carroll, R.C. and Gerrard, L.M.: Phosphorylation of platelet actin-binding protein during platelet activation. *Blood* **59**, 466-471, 1982.
34. Fox, J.E.B. and Phillips, D.R.: Role of phosphorylation in mediating the association of myosin with the cytoskeletal structure of human platelets. *J. Biol. Chem.* **257**, 4120-4126, 1982.
35. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishizuka, Y., Tamura, A. and Fujii, T.: A role of membrane in the activation of a new multifunctional protein kinase system. *J. Biochem.* **86**, 575-578, 1979.
36. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y.: Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* **254**, 3692-3695, 1979.
37. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y.: Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* **255**, 2273-2276, 1980.
38. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. and Nishizuka, Y.: Phospholipid turnover as a possible transmembrane signal for protein phosphorylation during human platelet activation by thrombin. *Biochem. Biophys. Res. Commun.* **97**, 309-317, 1980.
39. Gorman, R.R., Wierenga, W. and Miller, O.V.: Independence of the cyclic AMP-lowering activity of thromboxane A_2 from the platelet release reaction. *Biochim. Biophys. Acta* **572**, 95-104, 1979.
40. Miyahara, M., Aono, K., Queseda, J.S., Shimono, K., Baba, Y. and Yamashita, S.: Protection by cepharanthine of the mitochondrial function from damage induced by snake venom, phospholipase A_2, lysolecithin and lead. *Cell Struct. Funct.* **3**, 61-65, 1978.
41. Aono, K., Shiraishi, N., Arita, T., Inouye, B., Nakazawa, T. and Utsumi, K.: Changes in mitochondrial function by lipid peroxidation and their inhibition by biscoclaurine alkaloid. *Physiol. Chem. Physics* **13**, 137-144, 1981.
42. LeBreton, G.C., Dinerstein, R.J., Roth, L.J. and Feinberg, H.: Direct evidence for intracellular divalent cation redistribution associated with platelet shape change. *Biochim. Biophys. Res. Commun.* **71**, 362-370, 1976.

43. Skaer, R.J., Peters, P.D. and Emmines, J.P.: The localization of calcium and phosphorus in human platelets. *J. Cell Sci.* **15**, 679-692, 1974.
44. Gerrard, J.M., Schollmeyer, J.V. and White, J.G.: The role of contractile proteins in the function of the platelet surface membrane. In *Cytoskeletal Elements and Plasma Membrane Organization*, ed. G. Poste and G. Nicolson, North-Holland Publishing Co., New York, pp. 216-251, 1981.
45. Bell, R.L., Kennerly, A.D., Stanford, N. and Majerus, P.W.: Diglyceride lipase: A pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238-3241, 1979.
46. Kanaho, Y., Sato, T. and Fujii, T.: Mechanism of the inhibitory effect of cepharanthine of the aggregation of platelets. *Cell Struct. Funct.* **7**, 39-48, 1982.
47. Fujimoto, T., Ohara, S. and Hawiger, J.: Thrombin-induced exposure and prostacyclin inhibition of the receptor for factor Ⅷ/von Willebrand factor on human platelets. *J. Clin. Invest.* **69**, 1212-1222, 1982.
48. Graber, S. and Hawiger, J.: Evidence that changes in platelet cyclic AMP levels regulate the fibrinogen receptor on human platelets. *J. Biol. Chem.* **257**, 14606-14609, 1982.