Biochimica et Biophysica Acta, 986 (1989) 151–160
Elsevier

BBAMEM 74621

The aggregation of human platelet induced by ganodermic acid S

Chuen-Neu Wang [1], Jia-Chyuan Chen [2], Ming-Shi Shiao [3] and Cheng-Teh Wang [1]

[1] *Institute of Life Science, National Tsing Hua University, Hsinchu;* [2] *Laboratory of Electron Microscopy, College of Science, National Taiwan University, Taipei;* [3] *Department of Medical Research, Veterans General Hospital, Taipei, Taiwan (China)*

(Received 12 April 1989)
(Revised manuscript received 30 June 1989)

Key words: Platelet membrane; Ganodermic acid S; Aggregation; Phosphoinositide metabolism; Electron microscopy; (Human blood)

Incubation of gel-filtered human platelets in ganodermic acid S (lanosta-7,9(11),24-trien-3β,15α-diacetoxy-26-oic acid) showed that within a min 80% of the agent was taken up by the cells. The process of uptake was a simple diffusion, and the partition coefficient was about 10^5. The agent caused platelet aggregation at a concentration above 20 μM. Above the threshold, the extent of cell aggregation was in a linear relationship to the agent concentration. Also, the % of cell aggregation was comparable to the elevation of: (1) cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$); (2) protein phosphorylation; and (3) serotonin release. Also, it was correlated with the change in the interconversion of phosphoinositides. Moreover, platelets in various concentrations of ganodermic acid S appeared to show different time-course profiles in the changes of $[^{32}P]$phosphoinositides and $[^{32}P]$phosphatidic acid (PA). Upon addition of the agent, platelets showed an initial increase in all of the $[^{32}P]$phosphoinositides, and then the level of each kind of phosphoinositide decreased sequentially in phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol (PI). Below the aggregation threshold, platelets showed neither the resynthesis of $[^{32}P]PIP_2$ and $[^{32}P]PIP$ nor the accumulation of $[^{32}P]PA$. However, at 25 and 50 μM, platelets showed not only the resynthesis of $[^{32}P]PIP_2$ and $[^{32}P]PIP$ but also the accumulation of $[^{32}P]PA$. Interestingly, at 100 μM ganodermic acid S, platelets did not show the resynthesis of $[^{32}P]PIP_2$ and $[^{32}P]PIP$. In this case, the level of $[^{32}P]PA$ accumulation and that of $[^{32}P]PI$ decrease were less than those found in platelets at 50 μM ganodermic acid S. The results suggested that ganodermic acid S caused the activation of PIP_2 hydrolysis. Scanning electron microscopy (scanning EM) revealed that the morphology of platelets below the aggregation threshold appeared to be spiculate discoid shape. Above the threshold, the cells rounded up to spiculate irregular forms, which showed an elongation of filopodia after prolonged 30-s incubation. In addition, platelets at ≥ 50 μM ganodermic acid S showed the occurrence of membrane vesiculation. Hence, the incorporation of ganodermic acid S into platelet membrane resulted in the change of membrane morphology.

Introduction

Platelets play an important role in thrombosis and haemostasis [1]. The aggregation of platelets occurs when the cells respond to agonist stimulation. This phenomenon requires metabolic energy, calcium ion, fibrinogen, and the change of membrane structure so that platelets can adhere together [2]. In the cell, biochemical events of the stimulus-response coupling involve: (1) the onset of membrane event in either the production of thromboxanes or an increased turnover of phosphoinositides [3–5]; (2) an elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [6]; and (3) protein phosphorylation [7]. In thrombin-stimulated platelet aggregation, the onset of phosphoinositide metabolism is the primary membrane event leading to the cell aggregation [4,8–11]. Tysnes et al. [8] have pointed out that a preincubation of ^{32}P-labeled platelets at 37°C for 60 min is necessary to obtain the comparable results in the change of $[^{32}P]PI$ radioactivity to the change in quantity. Mauco et al. [12] have also indicated that different PI pools exist in platelets.

The stimulus-response of platelets also results in the rapid change of cell shape in less than half a minute [13]. The initially flat discoid cells round up into irregular form with the protrusion of pseudopods, which is

Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; scanning EM, scanning electron microscopy; $[Ca^{2+}]_i$, concentration of cytosolic free calcium ion.

Correspondence: C.-T. Wang, Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30043, China.

resulted from the reorganization of cytoskeletal molecules [14–17]. However, the morphology of a cell is also governed by its membrane morphology which includes both lipid bilayer and membrane skeleton [18–21]. It has been reported that membrane-acting agents can change platelet membrane morphology to either a sphere or spiculate form [21,22]. The bilayer couple hypothesis has been used to explain that the differential change in morphology is due to the different asymmetric distribution of these solutes between the bilayer leaflets [21].

Ganodermic acid S (lanosta-7,9(11),24-trien-3β,15α-diacetoxy-26-oic acid) is the major triterpenoid purified from *Ganoderma lucidum* (Fr.) Karst., which is a widely used Polyporaceae in traditional Chinese medicine [23]. It has been reported that the structure analogue of ganodermic acid S can lower the cellular content of cholesterol as well as inhibit the growth of cultured hepatoma cells [24,25]. Ganodermic acid S is an amphipathic molecule with a chemical structure similar to the detergents cholate and deoxycholate. We have shown that the infiltration of deoxycholate into platelet membrane results in the inhibition of platelet function [22]. Ganodermic acid S may be a membrane-acting agent as well. Hence, the effect of ganodermic acid S on human platelets was investigated.

In this study, three aspects of the effect of ganodermic acid S on human gel-filtered platelets were investigated. These were: (1) the uptake of ganodermic acid S by platelets; (2) the effects on platelet biochemical events as compared to those of thrombin stimulation; and (3) the effect on cell morphology as revealed by scanning electron microscopy (scanning EM). The results showed that ganodermic acid S was highly soluble in platelets membrane, hence causing membrane spiculation and vesiculation. The agent also activated PIP_2 hydrolysis which resulted in platelet aggregation.

Experimental procedures

Materials

Chemicals, organic solvents, thin-layer chromatographic plate (TCL plate silica gel 60), Omni-szintisol (cocktail) and EDTA (Titriplex II) were purchased from E. Merk (Darmstadt, F.R.G.). All organic solvents were redistilled before use. Both bovine thrombin and indomethacin were obtained from Sigma (St. Louis, MO, U.S.A.). Fura-2/AM was from Boehringer Mannheim (Mannheim, F.R.G.). Sepharose 2B was from Pharmacia (Piscataway, NJ, U.S.A.). X-ray film was purchased from Fuji Photo Film Co. (Japan). The carrier free [^{32}P]phosphate (2 mCi/ml, pH 7.4) was a generous gift from the Department of Radioisotope, National Tsing Hua University, Hsinchu, Taiwan, China.

Methods

Preparation of gel-filtered and ^{32}P-labeled platelets. Human platelets were isolated from freshly drawn blood of the healthy donors. The blood was anticoagulated with 10% (v/v) of 0.11 M sodium citrate. After a centrifugation (250 × *g* for 15 min) at room temperature, platelet-rich plasma was filtered through a Sepharose 2B column (2.5 × 20 cm) according to the method of Lages et al. [26]. The buffer solution was a modified calcium-free Hepes Tyrode's buffer containing 0.1% dextrose and 5 mM Hepes (pH 7.4). For preparing ^{32}P-labeled platelets, 20 ml of platelet-rich plasma was incubated with 2 mCi of carrier-free [^{32}P]phosphate at 37°C for 1 h prior to gel filtration. The cell number was estimated in a hemacytometer by a phase contrast microscope (Nikon, Type 104, Japan). In this study, isolated platelets were preincubated at 37°C for 60 min prior to further investigation. The preincubation was to restore the cells back to discoid shape [27]. Also, in this condition, changes in the radioactivities of [^{32}P] phosphoinositides and [^{32}P]PA might represent the changes in quantity during a 2-min study of phosphoinositide turnover [8].

Estimation of the uptake of ganodermic acid S by platelets. Platelets ($3 \cdot 10^8$ cells/ml) were incubated with various concentrations of ganodermic acid S. After a certain period, an aliquot was centrifuged through a layer of 0.5 ml silicon oil in a Laboratory centrifuge (Sigma, Model 202 CM, F.R.G.) at 13500 × *g* for 1 min. The supernatant, so called the first supernatant, was taken to measure the remained concentration of ganodermic acid S by a spectrophotometer at 243 nm (Beckman, Model DU-70, U.S.A.). The absorption coefficient of ganodermic acid S at 243 nm is 16600. Silicon oil had no effect on the extinction coefficient of ganodermic acid S. The uptake of ganodermic acid S by platelets was calibrated from a difference between the original concentration and the concentration left in the supernatant. In the condition of platelets at ≥ 50 μM ganodermic acid S, a second-step centrifugation was performed on the first supernatant, which was recentrifuged at 150000 × *g* for an hour in an ultracentrifuge (Beckman, Model L7, U.S.A.). The second supernatant was re-examined for the concentration of ganodermic acid S as above, while the pellet was examined by negative-staining electron microscopy.

Morphological studies by electron microscopy. Negative-staining electron microscopy was employed to examine the pellet obtained from the above described two-step centrifugation. The pellet was resuspended in 0.1 ml of Hepes Tyrode's buffer. A drop of sample was put on a carbon-coated Formvar grid (300 mesh). After a negative staining with 2% phosphotungstic acid for 30 s, the sample was examined in a Hitachi H-600 transmission electron microscope at 75 kV.

The sample preparation for scanning EM study was detailed in a previous study [27]. In brief, the agent-treated platelets were added with 5 vol. of ice-cold 2.5% glutaraldehyde in the modified calcium-free Hepes

Tyrode's buffer (pH 7.4). After a storage in ice for 1 h, the prefixed sample was then postfixed with 1% OsO_4 and dehydrated. The sample was dried in a critical-point dryer (Hitachi, Model HCP-2, Japan) in CO_2, and then plated with gold in an ion coater (Eiko Engineering, Model IB-2, Japan). The plated sample was observed under a Hitachi S-520 scanning electron microscope at 20 kV.

Analyses of biochemical events. The extent of platelet aggregation was observed in an aggrecorder (Daiichi, Model PA-3210, Japan). 0.5 ml of prewarmed platelets ($3 \cdot 10^8$ cells/ml) were added with various concentrations of ganodermic acid S. Control experiment was the aggegation under the stimulation by bovine thrombin (0.1 unit/ml). In the aggregation study, two inhibitors were also used, i.e. 2.5 mM EDTA and 10 μM indomethacin.

The study of protein phosphorylation was carried out by a modification of that described by Sano et al. [7]. Prewarmed ^{32}P-labeled platelets were stirred at 37°C in an aggrecorder, and then added with various concentrations of ganodermic acid S. After 1 min, a 45 μl aliquot was quenched with 10 μl of a sample buffer containing 8% dodecylsulfate (SDS), 20% 2-mercaptoethanol, 40% glycerol and 0.25 M Tris-HCl (pH 6.8). The mixture was boiled in a water bath for 3 min, and then separated by an electrophoresis in a 11% polyacrylamide gel containing 0.1% SDS as described by Laemmli [28]. The gel was stained with Coomassie brilliant blue, and dried in a slab dryer (Bio-Rad, Model 483, U.S.A.). The dried gel was subjected to autoradiography (Fuji X-ray film). Intensities of both 47-kDa and 20-kDa proteins were quantitated by a Hoefer scanning densitometer (Model GS 300, U.S.A.). The intensity used as 100% for each of the phosphorylated proteins was that obtained from the control experiment of thrombin stimulation.

Fura-2/AM was employed as a probe in the estimation of the change in $[Ca^{2+}]_i$. The experiment was performed according to the method of Pollock et al. [29] with some modifications. Platelet-rich plasma was incubated with 1 μM fura-2 acetoxymethyl ester at 37°C for 45 min, and then was filtered through a Sepharose 2B column as described previously. Prewarmed fura-2-loaded platelets were transferred into a prewarmed siliconized-quartz cuvette, finally, various amounts of ganodermic acid S were added. Fluorescence was measured in a fluorescence spectrophotometer (Hitachi, Model 654-60, Japan) at an excitation wavelength of 339 nm and an emission wavelength of 500 nm [30]. $[Ca^{2+}]_i$ was calibrated according to the method of Pollock et al. [29].

Secretion of serotonin was assayed as that described by Drummond and Gordon [31]. The 100% serotonin release was the value of platelet stimulated by 0.1 unit/ml bovine thrombin.

Analysis of phospholipid turnover. Analysis of the changes in [^{32}P]phosphoinositides and [^{32}P]PA was performed according to that described by Holmsen et al. [4]. ^{32}P-labeled gel-filtered platelets ($3 \cdot 10^8$ cells/ml) were incubated with various concentrations of ganodermic acid S. At each interval, an aliquot was taken for lipid extraction at 0°C in a solution containing 4 parts of chloroform/methanol/conc. HCl (20 : 40 : 1, v/v), 1 part of chloroform, and 1 part of water. Extracted lipid was separated by one-dimensional thin-layer chromatography on a precoated silica-gel 60 plate. The solvent system was: chloroform/methanol/methylamine (40% aqueous solution)/water (60 : 35 : 5 : 5, v/v). The chromatograms were visualized by an overnight autoradiography (Fuji X-ray film). Each ^{32}P-phospholipid species was scrapped into a vial, and added with 2 ml of Omni-szintisol (E. Merk). Radioactivity of each lipid species was counted in a scintillation counter (Beckman, Model LS-1801, U.S.A.).

Results

The uptake of ganodermic acid S by human gel-filtered platelets

Fig. 1A depicts the time course of the incorporation of ganodermic acid S into platelets. The results showed that: (1) the uptake of ganodermic acid S was saturable in 1 min; and (2) 80% of the molecule was taken up from the medium. In the condition of platelets at ⩾ 50 μM ganodermic acid S, the time-course profiles were different between the one obtained from the single-step centrifugation and the other from the two-step centrifugation. This difference was due to the membrane vesiculation occurring in platelets at ⩾ 50 μM ganodermic acid S. In this condition, a small size of pellet was obtained from the two-step centrifugation. The pellet, as revealed by a negative-staining electron microscopy, consisted of both vesicles (0.05–0.2 μm in diameter) and aggregates of membrane fragments (Fig. 1B). A plot of the uptake rate vs. external concentration of ganodermic acid S appeared to be linear (inset of Fig. 1A). Hence, the uptake process of ganodermic acid S by platelets was a simple diffusion. If one assumes that the size of platelets in the population was homogeneous as a discoid shape with 3 μm in diameter and 0.5 μm in height, then the ratio was about $3 \cdot 10^3$ for the agent concentration in platelet to that remained in aqueous phase. Therefore, the partition coefficient of ganodermic acid S in platelet membrane should be several hundred folds of $3 \cdot 10^3$.

Aggregation of gel-filtered platelet induced by ganodermic acid S

Platelets appeared to aggregate in ganodermic acid S of above 20 μM (Table I). Above the threshold concentration, a shape-change period appeared prior to the aggregation. The extent of platelet aggregation was in a

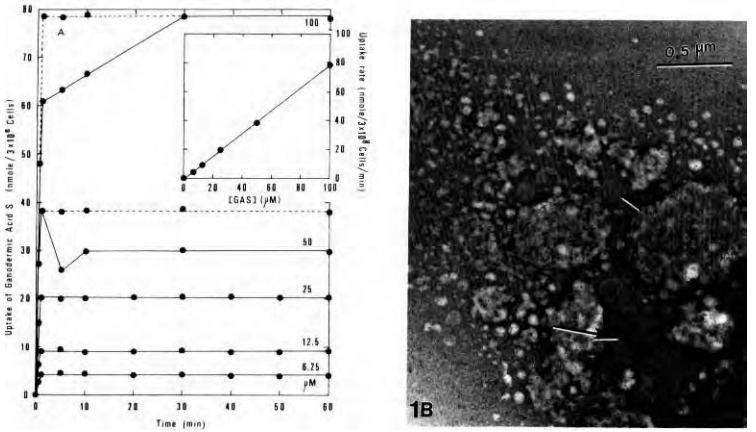

Fig. 1. (A) Time-course of the uptake of ganodermic acid S (GAS) by gel-filtered platelets. Prewarmed platelets ($3 \cdot 10^8$ cells/ml) were incubated with various concentrations of ganodermic acid S. The values of ganodermic acid S (in μM) are indicated in the figure. Quantification of ganodermic acid S was detailed in the Experimental procedures. Solid lines represent the data obtained from the first supernatant after a centrifugation at $13500 \times g$ for 1 min. Dashed lines represent the data obtained from the second supernatant after a two-step centrifugation at $150000 \times g$ for 1 h. The inset shows a plot of the uptake at the first min vs. the concentration of ganodermic acid S. Data points were taken from an average of at least six separate experiments. (B) Electron micrograph of a negative staining pellet obtained from a two-step centrifugation of platelets at 100 μM ganodermic acid S for 1 min ($\times 45000$). Arrow and arrow head indicate the vesicles (0.05–0.2 μm in diameter) and the aggregates of membrane fragments, respectively. Details of the experiment were given in Experimental procedures.

linear relationship to the concentration of ganodermic acid S. At 75 μM, platelets aggregated in a similar profile as that under the stimulation of thrombin. The aggregation was inhibited in the presence of 2.5 mM EDTA, but not in 10 μM indomethacin. It indicates that platelet activation by ganodermic acid S did not require the thromboxane pathway.

Several biochemical events of platelets activation by ganodermic acid S were further investigated by comparison with those of thrombin stimulation (Fig. 2). The results showed that platelets in various concentrations of ganodermic acid S appeared to have a concentration-dependent increase in the level of: (1) $[Ca^{2+}]_i$, (2) phosphorylation of 47K and 20K proteins, and (3) serotonin release. These indicate that the concentration-dependent aggregation was due to platelet population heterogeneity. Also, it implies that the incorporation of ganodermic acid S into platelet membrane might activate the turnover of phosphoinositides.

Effect on the metabolism of phosphoinositides by ganodermic acid S

The concentration effect of ganodermic acid S on the turnover of phosphoinositides was investigated by comparison with the turnover under thrombin stimulation. Upon the addition of ganodermic acid S, the initial 2-min time-course was followed in the change of radioactivity of each kind of phosphoinositide and that of PA (Fig. 3). Under thrombin stimulation, the time-course profiles showed: (1) a gradual decrease in [^{32}P]PI (Fig. 3A); (2) a gradual increase in [^{32}P]PIP up to 1 min (Fig. 3B); (3) an initial decrease in [^{32}P]PIP_2 with an increase in the level after a 10-s incubation (Fig. 3C); and (4) a continuous increase in [^{32}P]PA (Fig. 3D). Interestingly, the time-course profiles of platelets in ganodermic acid S appeared to be different from those of control. In addition, the time-course patterns of platelets at below the aggregation threshold were different from those found at above the threshold. Specifically, (1) in the change of [^{32}P]PI (Fig. 3A), platelets at various concentrations of ganodermic acid S all showed an initial increase in the level with a decrease at a prolonged incubation. The rate of decrease was dependent on the agent concentration. Interestingly, the decreasing rate of [^{32}P]PI for platelets at 100 μM was slower than those found at 25 and 50 μM. (2) In the change of [^{32}P]PIP (Fig. 3B), platelets at below the threshold showed an increase in [^{32}P]PIP up to 20 s,

TABLE I

Aggregation of human platelets at various concentrations of ganodermic acid S

Data (mean ± S.D.) were taken from at least six experiments. n.d., not detectable. Control stands for the aggregation under stimulation of 0.1 unit/ml of bovine thrombin.

Ganodermic acid S (μM)	Shape-change period [a] (s)	Initial aggregation rate [b] (%/min)	% of cell aggregation [c]
Control	4.8 ± 0.2	76.3 ± 7.4	94.2 ± 7.9
6.25	n.d.	n.d.	0
12.5	n.d.	n.d.	0
25	26.7 ± 3.2	13.2 ± 0.5	11.1 ± 0.4
50	21.3 ± 3.5	70.5 ± 2.3	56.3 ± 1.5
100	14.4 ± 2.7	98.0 ± 4.4	96.0 ± 3.0
100 + EDTA [d]	14.0 ± 2.5	17.2 ± 0.5	20.5 ± 1.1
100 + indomethacin [d]	22.4 ± 3.6	95.7 ± 4.5	97.8 ± 4.1

[a] The shape change period represents the time between addition of the agent and appearance of the minimum transmission observed in an aggrecorder.

[b] The initial aggregation rate (%/min) was calculated from the initial slope of aggregation curve, and represented the percentage of platelet aggregation per min.

[c] The % of cell aggregation was taken from the cell aggregation in 10 min.

[d] The gel-filtered platelets were preincubated with either 2.5 mM EDTA or 10 μM indomethacin for 5 min, then were added with 100 μM ganodermic acid S.

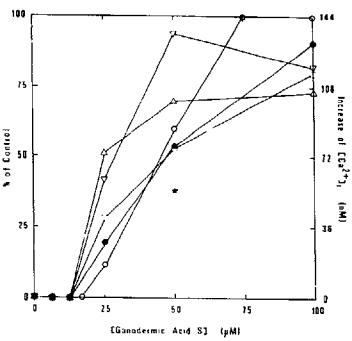

Fig. 2. Concentration effect of ganodermic acid S on gel-filtered platelets in the changes of $[Ca^{2+}]_i$ (●); phosphorylation of protein 20K (△) and 47K (▽); serotonin release (□) and aggregation (○). Either gel-filtered or ^{32}P-labeled platelets ($3 \cdot 10^8$ cells/ml) were incubated with various concentrations of ganodermic acid S for 1 min. Details of the experiments were given in Experimental procedures. 100% of protein phosphorylation, serotonin release, and cell aggregation were the values obtained from the control experiments under thrombin stimulation. The star (✱) in the center of the figure represents the $[Ca^{2+}]_i$ increase in the control experiment. The data points were taken from an average of at least six separate experiments.

and then a gradual decrease appeared. However, at above the threshold, an initial increase of [^{32}P]PIP appeared up to 10 s with the appearance of a drastic decrease in [^{32}P]PIP. Then, another increase of [^{32}P]PIP showed up at 20 s and 40 s for platelets at 25 μM and 50 μM ganodermic acid S, respectively. Surprisingly, the resynthesis of [^{32}P]PIP did not occur to platelets at 100 μM in which [^{32}P]PIP leveled off at 40 s. (3) In the change of [^{32}P]PIP_2 (Fig. 3C), platelets below the threshold showed an increase in the level with a gradual decrease after 10 s. On the contrary, the cells at 25 and 50 μM showed an immediate decrease in [^{32}P]PIP_2 till 20 s and 40 s, respectively. After then, an increase in [^{32}P]PIP_2 occurred. Again, the resynthesis of [^{32}P]PIP_2 did nto occur to platelets at 100 μM. (4) Platelets at below the threshold showed no significant change in [^{32}P]PA (Fig. 3D). However, above the threshold, the cells showed several-fold increase in [^{32}P]PA. Interestingly, at the first 10 s, the rate of increase in [^{32}P]PA was slower than that increase observed under thrombin stimulation. After 10 s incubation, the rate of increase in [^{32}P]PA was faster than that in thrombin. After 2 min incubation, the level of [^{32}P]PA in platelets at 25 μM was half of that in the cells at 50 μM. However, the level of [^{32}P]PA in platelets at 100 μM appeared to be 60% of that at 50 μM.

In summary, these results indicate that ganodermic acid S immediately caused an increase in the level of each kind of phosphoinositide, and then the activation of PIP_2 hydrolysis followed. This kind of effect by ganodermic acid S was a concentration-dependent phenomenon.

Morphological changes of platelet in ganodermic acid S

Since platelets were saturable with ganodermic acid S in 1 min, scanning EM was employed to observe the morphological changes of platelets incubated for 1 min at various concentrations of the agent (Fig. 4). For each concentration, platelets appeared to show the same morphological change between the incubations of the cells at 37 °C for 30 and 60 min. Platelets below the aggregation threshold changed the morphology to spiculate discoid shape even though an aggrecorder did not detect the change (Fig. 4B and C). The width of a filopodium was about 70 nm. Above the threshold, platelet either changed the morphology to spiculate discoid shape or rounded up to spiculate irregular form (Figs. 4D and 4E). The latter form appeared to be more in the population when the cells were incubated at a higher concentration of ganodermic acid S (Figs. 4D, 4E and 5). In addition, in a higher concentration of

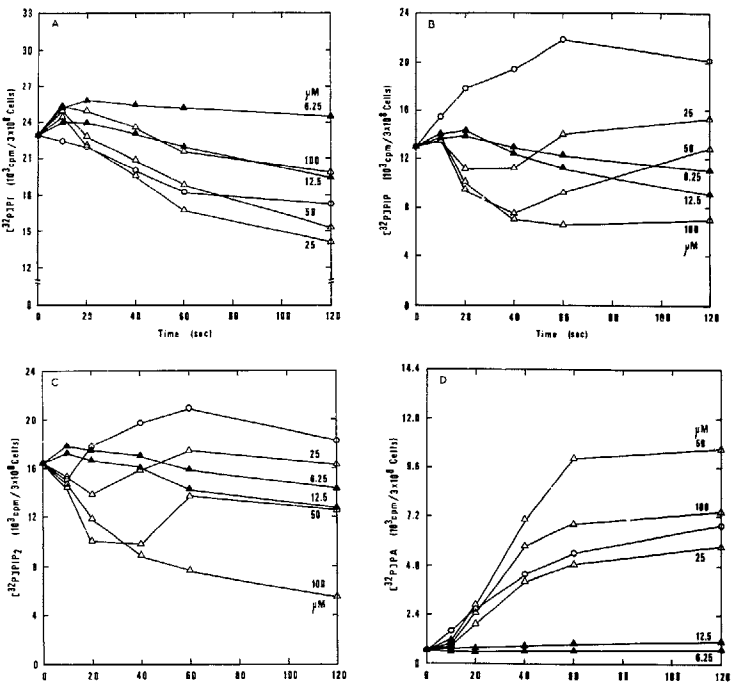

Fig. 3. Time-courses of the effect of ganodermic acid S on ^{32}P-labeled platelets in the changes of [^{32}P]PI (A), [^{32}P]PIP (B), [^{32}P]PIP$_2$ (C), and [^{32}P]PA (D). ^{32}P-labeled platelets were incubated at 37 °C for 1 h, and then added with various concentrations of ganodermic acid S. Experiments were detailed in Experimental procedures. The concentrations of ganodermic acid S (in μM) are indicated in the figures. Solid triangles (▲) represent the changes in each kind of [^{32}P]phospholipid at below the aggregation threshold concentration of ganodermic acid S. Open triangles (△) represent that changes at above the aggregation threshold concentation of ganodermic acid S. Open circles (○) represent the changes under thrombin stimulation. The data points were taken from an average of at least six separate experiments.

ganodermic acid S, each deformed cell appeared to have a higher number of filopodia, which were longer in length (Figs. 4D and 4E).

The sequentially morphological change of platelets at 100 μM ganodermic acid S was further compared with that change in thrombin (Fig. 5). Upon the addition of ganodermic acid S for 10 s, platelets deformed to a mixed population of spiculate discoid; spiculate irregular form; and the spiculate discoid with small blebs on the cell surface (Fig. 5A). In the maximum shape-change stage, i.e. at 20 s incubation, the cells changed the morphology to irregular forms with enlarged blebs plus an increased number of filopodia per cell (Fig. 5C). At the onset of aggregation (30 s incubation), the cells all rounded up to spiculate irregular form in which the number of filopodia were higher than that found at 20 s incubation (Fig. 5E). Also, vesicles (200 nm in diameter) apepared at this stage. The length of each filopodium

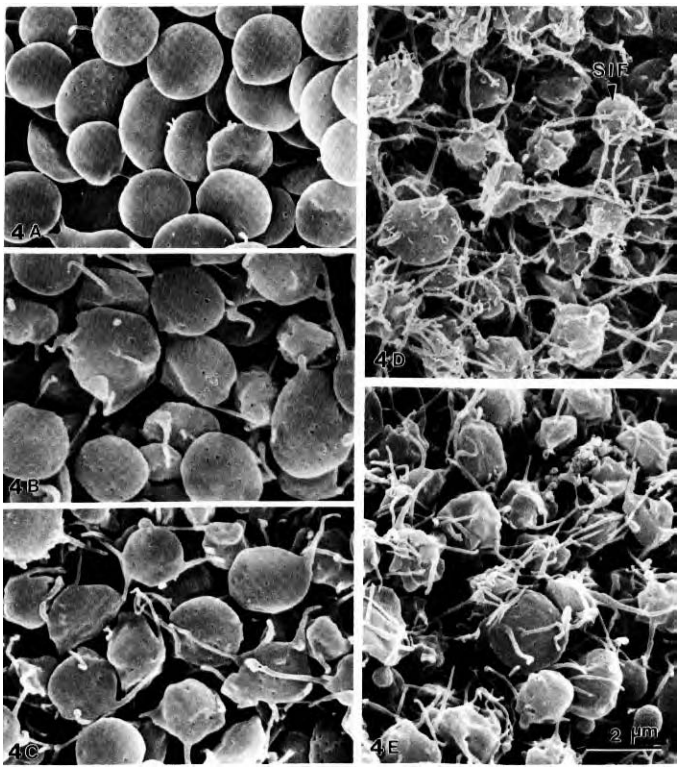

Fig. 4. Scanning electron micrographs of gel-filtered platelets in various concentrations of ganodermic acid S (×9000). Gel-filtered platelets were preincubated at 37 °C for 60 min (A), and then incubated for 1 min with ganodermic acid S of 6.25 μM (B), 12.5 μM (C), 25 μM (D), and 50 μM (E). The abbreviation in parenthesis indicates the cell in spiculate irregular form (SIF). Details of experiments are given in Experimental procedures.

became longer at 1 min (Fig. 5G). In the control experiment of thrombin stimulation, the whole cell population changed to irregular form with protrusion of pseudopods (width > 200 nm) (Fig. 5B). The cells attached to each other through pseudopods and then clamped together (Figs. 5D, 5F, 5H). In this case, the cell surface showed no filopodium. Hence, the formation of filopodia was a characteristic phenomenon induced by ganodermic acid S.

Discussion

The study demonstrates that ganodermic acid S incorporates rapidly into platelet membrane resulting in

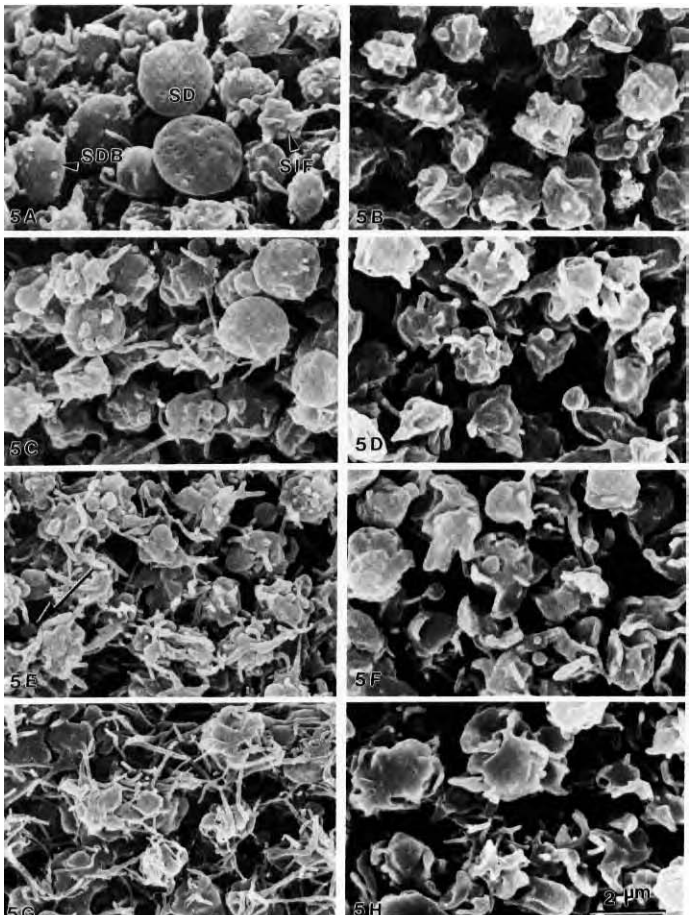

Fig. 5. Scanning electron micrographs of the sequentially morphological changes of gel-filtered platelets in 100 μM ganodermic acid S and bovine thrombin (×9000). The prewarmed platelets were incubated with either 100 μM ganodermic acid S for 10 s (A); 20 s (C); 30 s (E); and 1 min (G), or thrombin (0.1 unit/ml) for 10 s (B); 20 s (D); 30 s (F); and 1 min (H). In the figure, a filopodium had a width of 70 nm, while a pseudopod had a width > 200 nm. The arrow in E indicates a vesicle (0.2 μM in diameter). The abbreviations in parentheses indicate the spiculate discoid cell (SD), spiculate irregular form (SIF), and spiculate disc with blebs (SDB). Details of experiments are given in Experimental procedures.

the membrane spiculation and vesiculation (Figs. 1B, 4, and 5). The agent exerts a biphasic concentration effect on platelet aggregation (Table I, Fig. 2). It causes the activation of PIP_2 hydrolysis (Fig. 3).

The study indicates that ganodermic acid S is a membrane-acting agent, since: (1) the uptake by platelet is saturable in 1 min (Fig. 1A); (2) the agent is highly soluble in platelet membrane with a partition coefficient of about 10^5; and (3) it causes platelet membrane spiculation and vesiculation (Figs. 1B, 4, 5). This kind of change in membrane morphology also occurs to platelets in anionic detergents dodecylsulfate and deoxycholate [22]. According to the explanation of the bilayer couple hypothesis, the spiculation of platelet membrane may mean that the distribution of ganodermic acid S in membrane bilayer is more in the outer leaflet than in the inner one [21,22]. Although ganodermic acid S is an anionic amphiphile with a complicated ring structure resembling that of deoxycholate [32], it appears that platelets only aggregate in response to ganodermic acid S but not to deoxycholate [22]. Hence, ganodermic acid S may exert some effect on platelet membrane proteins.

The study by an aggregometer shows that platelet aggregation occurs in ganodermic acid S at the concentration higher than 20 μM (Table I). Below the threshold, platelets do not show the shape change detected under an aggrecorder (Table I). However, scanning EM reveals that platelets change the morphology to spiculate discoid shape (Figs. 4B and 4C). Therefore, the measurement of turbidimetric shape change of platelets by an aggrecorder is not sensitive enough to detect the formation of filopodia on platelet cell surface. This confirms a previous suggestion by Milton and Frojmovic [33]. Above the threshold, the agent can activate PIP_2 hydrolysis, which results in an increase in the level of: (1) $[Ca^{2+}]_i$, (2) protein phosphorylation, and (3) serotonin release (Fig. 2). In this study, the result of serotonin release may represent its secretion from dense granule. Since EDTA can inhibit the aggregation induced by ganodermic acid S, it means that the release of Ca^{2+} from dense granule occurs under the effect of ganodermic acid S. In addition, the effect of agent on the metabolism of phosphoinositides was studied by using ^{32}P-labeled platelets which were preincubated at 37°C for 60 min. In this condition, the control experiment of thrombin stimulation shows the comparable results as those reported from Tysnes et al. [8]. Hence, the changes in the radioactivities of phosphoinositides and PA may represent their changes in quantity during the 2-min study of phosphoinositide turnover. The study indicates that the incorporation of ganodermic acid S into platelet membrane may exert three kinds of effect on the interconversion of phosphoinositides. Firstly, the infiltration of ganodermic acid S into platelet membrane immediately causes an elevation of [^{32}P]PI from the other PI pool before the activation of PIP_2 hydrolysis occurs, since platelets in ganodermic acid S show an initial elevation of each kind of [^{32}P]phosphoinositide (Fig. 3). This effect is also observed in platelets below the aggregation threshold. Although platelets below the threshold show the decreases in [^{32}P]PI, [^{32}P]PIP and [^{32}P]PIP_2, the cells do not show either the resynthesis of [^{32}P]PIP_2 and [^{32}P]PIP or the change of [^{32}P]PA, $[Ca^{2+}]_i$, and protein phosphorylation. In this situation, the agent may not turn on the phosphoinositide interconversion to produce a concentration of both inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol high enough to cause platelet aggregation. Secondly, the phosphoinositide interconversion is turned on in the condition of platelets at either 25 or 50 μM ganodermic acid S, since the cells all show: (1) the resynthesis of both [^{32}P]PIP, and [^{32}P]PIP; and (2) the elevation in [^{32}P]PA, $[Ca^{2+}]_i$ and protein phosphorylation. Thirdly, the interconversion of phosphoinositides may be partially blocked for platelets at a higher concentration as 100 μM ganodermic acid S, since in this case, the cells show: (1) no resynthesis of both [^{32}P]PIP_2 and [^{32}P]PIP occurred, and (2) the levels of both [^{32}P]PA accumulation and the [^{32}P]PI decrease are less than those happened in platelets at 50 μM. Therefore, the infiltration of various concentrations of ganodermic acid S into platelet membrane results in differential effect on the metabolism of phosphoinositides.

The study by scanning EM reveals that platelets below the aggregation threshold change the morphology to spiculate disc with no change in $[Ca^{2+}]_i$ (Figs. 2, 4B, 4C). Hence, the formation of filopodia in ganodermic acid S may be a direct effect by the agent. Above the threshold, the cells round up to spiculate irregular form (Figs. 4D, 4E and 5). This kind of change in cell volume is due to the elevation of $[Ca^{2+}]_i$, which resulting in the reorganization of cytoskeleton (Fig. 2) [34–41]. This is further supported by the study of sequentially morphological change of platelets at 100 μM ganodermic acid S. The study is comparable to the time-course studies of platelet aggregation and phosphoinositide interconversion (Figs. 3 and 5). The comparison shows that the incorporation of ganodermic acid S into platelet membrane instantly activates the hydrolysis of PIP_2, since after a prolonged 30-s incubation the cell morphology changes to the spiculate irregular form accompanied by cell aggregation. At prolonged 1 min incubation, the elongation of filopodia is due to the incorporation of more ganodermic acid S into the outer leaflet of platelet membrane. Hence, the membrane spiculation caused by ganodermic acid S is a separate phenomenon from platelet aggregation in response to ganodermic acid S.

In summary, the study shows that the insertion of

ganodermic acid S into platelets affects not only the platelet membrane morphology but also the metabolic pool of phosphoinositides in the plasma membrane.

Acknowledgments

This research was supported by a grant from the National Science Council of Republic of China (NSC78-0203-B007-03). We thank Messrs. Wei-Jern Tsai, Jehn-Yuu Lee and Young-Ji Shiao for their help on the work. The work is in a partial fulfillment of C.-N. Wang's doctoral dissertation.

References

1 White, J.G. (1983) in Measurements of Platelet Function (Harker, L.A. and Zimmerman, T.S., eds.), pp. 1–25, Churchill Livingstone, London.
2 Kinlough-Rathbone, R.L., Packham, M.A. and Mustard, J.F. (1983) in Measurements of Platelet Function (Harker, L.A. and Zimmerman, T.S., eds.), pp. 64–91, Churchill Livingstone, London.
3 Brass, L.F. and Joseph, S.K. (1985) J. Biol. Chem. 260, 15172–15179
4 Holmsen, H., Dangelmaier, C.A. and Rongved, S. (1984) Biochem. J. 222, 157–167.
5 Nishizuka, Y. (1986) Science 233, 305–312.
6 Berridge, M.J. and Irvine, R.F. (1984) Nature (London) 312, 315–321.
7 Sano, K., Takai, Y., Yamanishi, J. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010–2013.
8 Tysnes, O.-B., Verhoeven, A.J.M. and Holmsen, H. (1986) Biochim. Biophys. Acta 889, 183–191.
9 Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705–12708.
10 Wilson, D.B., Neufeld, E.J. and Majerus, P.W. (1985) J. Biol. Chem. 260, 1046–1051.
11 Steen, V.M., Tysnes, O.-B. and Holmsen, H. (1988) Biochem. J. 253, 581–586.
12 Mauco, G., Dajeans, P., Chap, H. and Douste-Blazy, L. (1987) Biochem. J. 244, 757–761.
13 Dereudeau, D.A. (1987) Trends Biochem. Sci. 12, 439–442.
14 Fox, J.E.B. (1987) in Thrombosis 11th Congress Haemostasis (Verstraete, M., Vermylen, J., Lijnen, H.R. and Arnout, J., eds.), pp. 175–225, Louvain University Press, Louvain.
15 Zucker, M.B. and Nachmias, V.T. (1985) Arteriosclerosis 5, 2–18.
16 Rink, T.J. and Hallam, T.J. (1984) Trends Biochem. Sci. 9, 215–219.
17 Wang, C.-T., Shiao, Y.-J., Chen, J.-C., Tsai, W.-J. and Yang, C.C. (1986) Biochim. Biophys. Acta 856, 244–258.
18 Kanaho, Y. and Fujii, T. (1982) Biochem. Biophys. Res. Commun. 106, 513–519.
19 Nachmias, V.T., Sullender, J.S. and Fallon, J.R. (1979) Blood 53, 63–72.
20 Ferrell Jr., J.E. and Huestis, W.H. (1984) J. Cell Biol. 98, 1992–1998.
21 Ferrell Jr., J.E., Mitchell, K.T. and Huestis, W.H. (1988) Biochim. Biophys. Acta 939, 223–237.
22 Shiao, Y.-J., Chen, J.-C. and Wang, C.-T. (1989) Biochim. Biophys. Acta 980, 56–68.
23 Shiao, M.-S. and Lin, L.-J. (1987) J. Nat. Prod. 50, 886–890.
24 Taylor, F.R., Saucier, S.E., Shown, E.P., Parish, E.J. and Kandutsch, A.A. (1984) J. Biol. Chem. 259, 12382–12387.
25 Toth, J.O., Luu, B. and Ourisson, G. (1983) Tetrahedron Lett. 24, 1081–1084.
26 Lages, B., Scrutton, M.C. and Holmsen, H. (1975) J. Lab. Clin. Med. 85, 811–821.
27 Tsai, W.-J., Chen, J.-C. and Wang, C.-T. (1988) Biochim. Biophys. Acta 940, 105–120.
28 Laemmli, U.K. (1970) Nature 227, 680–685.
29 Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) Biochem. J. 235, 869–877.
30 Cobbold, P.H. and Rink, T.J. (1987) Biochem. J. 248, 313–328.
31 Drummond, A.H. and Gordon, J.L. (1974) Thromb. Diath. Haemorrh. 31, 336.
32 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
33 Milton, J.G. and Frojmovic, M.M. (1984) J. Lab. Clin. Med. 104, 805–815.
34 Carroll, R.C., Butler, R.G., Morris, P.A. and Gerrard, J.M. (1982) Cell 30, 385–393.
35 Fox, J.E.B., Boyles, J.K., Reynolds, C.C. and Phillips, D.R. (1984) J. Cell Biol. 98, 1985–1991.
36 Chadwick Cox, A., Carroll, R.C., White, J.G. and Rao, G.H.R. (1984) J. Cell Biol. 98, 8–15.
37 Fox, J.E.B. and Phillips, D.R. (1983) Semin. Hematol. 20, 243–260.
38 Jennings, L.K., Fox, J.E.B., Edwards, H.H. and Phillips, D.R. (1981) J. Biol. Chem. 256, 6927–6932.
39 Steiner, M. and Ikeda, Y. (1979) J. Clin. Invest. 63, 443–448.
40 Gershman, L.C., Selden, L.A. and Estes, J.E. (1986) Biochem. Biophys. Res. Commun. 135, 607–614.
41 Oosawa, F., Asakura, S., Asai, H., Kasai, M., Kobayashi, S., Mihashi, K., Ooi, T., Taniguchi, M. and Nakano, E. (1964) in Biochemistry of Muscle Contraction (Gergely, J., ed.), pp. 158–172, Academic Press, New York.