The inhibition of human platelet function by ganodermic acids

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Human gel-filtered platelets aggregate at $> 20 \mu\text{M}$ -ganodermic acid S [lanosta-7,9(11),24-triene-3 β ,15 α -diacetoxy-26-oic acid] [Wang, Chen, Shiao & Wang (1989) *Biochim. Biophys. Acta* **986**, 151–160]. This study showed that platelets at $< 20 \mu\text{M}$ -ganodermic acid S displayed both concentration- and time-dependent inhibition of function, in which the agent potency in response to inducers was ADP-fibrinogen $>$ collagen $>$ thrombin. The agent caused a biphasic time-dependent effect on platelet phosphoinositide metabolism. The first phase involved the decrease in the pool size of phosphoinositide by 10–20%. The second phase, in which both the resynthesis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and the decrease of [³²P]phosphatidic acid occurred, took place after 30 min. Scanning electron microscopy also revealed a time-dependent morphological change in platelets in the presence of the agent. The cells initially became spiculate discs, then swelled to a 'potato-like' morphology at 60 min. Further studies on the time-dependent inhibition of thrombin response revealed that: (1) the percentage inhibition of cell aggregation was comparable with that occurring with an increase of cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) or the phosphorylation of marker proteins; (2) [³²P]P_i-labelled platelets showed the time-dependent inhibition of thrombin-stimulated PIP₂ resynthesis as indicated by first-2-min time-course studies of phosphoinositide interconversion; (3) scanning electron microscopy revealed that the aged platelet population showed an increase in the percentage of non-responding cells on prolonged incubation. The results, taken together, enabled one to discuss a possible mechanism for the time-dependent inhibition by ganodermic acid S of platelet response to thrombin.

INTRODUCTION

Platelets are important in thrombosis and haemostasis [1]. The cells aggregate under the stimulation of agonists such as thrombin, ADP-fibrinogen and collagen. Recently, we have shown that human platelets aggregate in ganodermic acid S [lanosta-7,9(11),24-triene-3 β ,15 α -diacetoxy-26-oic acid], which is the major oxygenated triterpenoid purified from *Ganoderma lucidum* (Fr.) Karst. (Polyporaceae), a fungus widely used in traditional Chinese medicine [2]. The aggregation of platelets appears at $> 20 \mu\text{M}$ -ganodermic acid S, which activates PIP₂ hydrolysis and turns on phosphoinositide interconversion [3]. Although platelets at $< 20 \mu\text{M}$ -agent do not aggregate, the cells change their morphology to spiculate discs. Similar morphological changes have been shown in platelets in the anionic detergents dodecyl sulphate and deoxycholate [4]. Platelets in these two detergents display an inhibited response to both ADP-fibrinogen and collagen. It was considered worthwhile to investigate whether the drug can affect platelet function.

Platelets deform either to a spiculate or a spherical form in various membrane-acting agents [4,5]. The changes in membrane morphology can be explained by the bilayer couple hypothesis [5,6]. Platelets in the anionic detergents dodecyl sulphate and deoxycholate display a sequential morphological change on prolonged incubation [4]. Ganodermic acid S is an anionic amphiphile. Platelets in the agent may, in addition, show a time-dependent morphological change.

This study firstly showed that platelets at $< 20 \mu\text{M}$ -ganodermic acid S exhibited an inhibited response not only to ADP-fibrinogen and collagen, but also to thrombin. The inhibitory effects were enhanced at 60 min incubation. This time-dependent inhibition by ganodermic acid S of platelet response to thrombin was

further investigated by: (1) measuring the effect on thrombin-stimulated phosphoinositide turnover, and (2) scanning electron microscopy to reveal the morphological change. These two results were collated to provide a possible explanation for the time-dependent inhibition by ganodermic acid of platelet response to thrombin.

EXPERIMENTAL

Materials

Chemicals, organic solvents, t.l.c. (silica-gel 60) plates, Omni-Szintisol (scintillation cocktail) and EDTA (Titriplex II) were purchased from E. Merck (Darmstadt, Germany). All organic solvents were redistilled before use. Bovine thrombin, apyrase, collagen (type III, from calf skin), ADP, human fibrinogen, NADH and phorbol 12-myristate 13-acetate were obtained from Sigma (St. Louis, MO, U.S.A.). Fura-2 acetoxymethyl ester (AM) was from Boehringer Mannheim (Mannheim, Germany). Sepharose 2B was from Pharmacia (Piscataway, NJ, U.S.A.). X-ray film was purchased from Fuji Photo Film Co. (Tokyo, Japan). Carrier-free [³²P]P_i (2 mCi/ml, pH 7.4) was generously given by the Department of Radioisotopes, National Tsing Hua University.

Preparation of gel-filtered and [³²P]P_i labelled platelets

Human blood was freshly drawn from the healthy donors and 10% (v/v) 0.11 M-sodium citrate (anticoagulant) was added. The method of Lages *et al.* [7] was used to obtain gel-filtered platelets suspended in a modified Ca²⁺-free Hepes Tyrode's buffer containing 0.1% dextrose and 5 mM-Hepes, pH 7.4. To study the response to ADP-fibrinogen, apyrase (4 $\mu\text{g}/\text{ml}$) was added to platelet-rich plasma just before gel filtration. For preparing

Abbreviations used: PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; DG, diacylglycerol; s.e.m., scanning electron microscopy; [Ca²⁺]_i, concentration of cytosolic free Ca²⁺; /AM, acetoxymethyl ester.

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^{32}P -labelled platelets, 20 ml of platelet-rich plasma was incubated with 2 mCi of carrier-free ^{32}P at 37°C for 1 h before gel filtration. The cell number was counted in a haemocytometer by means of a phase-contrast microscope (Nikon, type 104; Tokyo, Japan). The isolated platelets were preincubated at 37°C for 30 min before further investigation. The preincubation was to restore the cell back to a discoid shape [8]. Also, under these conditions, the changes in radioactivities of ^{32}P phosphoinositides and ^{32}P phosphatidic acid (^{32}P PA) may represent the quantitative changes during the 2 min study of phosphoinositide turnover [9].

Effects of a prolonged incubation in ganodermic acid S on platelet functions

Gel-filtered platelets (3×10^8 cells/ml) were incubated at 37°C in various concentrations of ganodermic acid S for a certain period. A portion (0.5 ml) of cell suspension was taken to study the effect of the agent on agonist-stimulated aggregation in an aggregometer (Daiichi, model PA-3220; Kyoto, Japan). The agonists were bovine thrombin (0.1 unit/ml), collagen (100 $\mu\text{g}/\text{ml}$), ADP (20 μM) plus fibrinogen (1 mg/ml) and phorbol 12-myristate 13-acetate (10 ng/ml). Under each condition a parallel control was performed to represent 100% of the extent of cell aggregation.

Analysis of the changes of both phospholipid mass and incorporation of ^{32}P into various phospholipid classes

^{32}P -labelled gel-filtered platelets (3×10^8 cells/ml) were incubated with various concentrations of ganodermic acid S. After various times two equal samples were taken to investigate: (1) the time-dependent changes in ^{32}P incorporation into each phospholipid class, and (2) the thrombin-stimulated 2-min time courses of the changes in ^{32}P phosphoinositides and ^{32}P PA.

Analysis of the radioactivity in each phospholipid class was performed as described by Holmsen *et al.* [10]. Lipid was extracted at 0°C in a solution containing 4 parts of chloroform/methanol/conc. HCl (20:40:1, by vol.), 1 part of chloroform and 1 part of water. Extracted lipid was separated by one-dimensional t.l.c. on a precoated silica-gel 60 plate. The solvent system was chloroform/methanol/methylamine (aq. 40% solution)/water (12:7:1:1, by vol.). Chromatograms were subjected to overnight autoradiography (Fuji X-ray film) to reveal labelled phospholipids. Each ^{32}P phospholipid class was scraped off the plate. Radioactivity was counted in a liquid-scintillation counter (Beckman, model LS-1801) by adding 2 ml of Omni-szintisol. Lipid mass was quantified by the method of Chalvardjian & Rudnicki [11].

Morphological studies by electron microscopy

Sample preparation for the scanning-electron-microscopical study was described previously [8]. In brief, platelets after incubation in ganodermic acid S for various periods were divided into two parts. One part was prepared for fixation. The second part was added to thrombin for another 1 min. The samples were added to 5 vol. of ice-cold 2.5% (v/v) glutaraldehyde in the modified Ca^{2+} -free HEPES Tyrode's buffer, pH 7.4. After storage on ice for 1 h, the prefixed sample was then post-fixed with 1% OsO_4 and dehydrated. The sample was dried in a critical-point dryer (Hitachi, model HCP-2), in CO_2 , and then plated with gold in an ion coater (Model IB-2; Eiko Engineering, Mito, Japan). The plated sample was observed under a Hitachi S-520 scanning electron microscope at 20 kV.

Other analyses

The effect of ganodermic acid S on the platelet protein phosphorylation as well as that on the changes in $[\text{Ca}^{2+}]_i$ was

Fig. 1. Differential inhibition of platelet functions during 60 min incubation of human platelets in ganodermic acid S

Gel-filtered human platelets (3×10^8 cells/ml) were incubated for a certain period in ganodermic acid S (GAS) at various concentrations (as indicated in the Figure), and then added with the agonists: (a) ADP (20 μM) plus fibrinogen (1 mg/ml); (b) collagen (100 $\mu\text{g}/\text{ml}$); and (c) bovine thrombin (0.1 unit/ml). In each condition, the percentage of cell aggregation was that at 10 min after the addition of the agonist. A parallel control experiment was also performed to give 100% response to each kind of agonist. The 100% response to ADP-fibrinogen, collagen, and thrombin corresponded to 60, 85 and 92% of the extent of cell aggregation, respectively. All data are expressed as means $\pm$ S.E.M. ($n = 6$).

detailed previously [3]. Protein phosphorylation was measured by a modification of the method described by Sano *et al.* [12]. Fura-2/AM was employed as a probe in the estimation of the change in $[Ca^{2+}]_i$. The experiment was performed by a modification of the procedure described by Pollock *et al.* [13]. The 'leakiness' of the marker in the supernatant was monitored by: (1) measuring the activity of lactate dehydrogenase in the supernatant by the method of Bergmeyer & Bernt [14], (2) determining Mg^{2+} by using an atomic-absorption spectrometer (Perkin–Elmer, model 5000), and (3) measuring $[^{32}P]ATP$ by the method of Holmsen *et al.* [15]. The supernatant used for these assays was obtained from centrifugation [laboratory centrifuge (Sigma, model 202 CM); 13500 g for 1 min] of the suspension in which platelets were treated with ganodermic acid S for a certain period.

RESULTS

Inhibition of platelet function by ganodermic acid S

Gel-filtered platelets at $< 20 \mu M$ -ganodermic acid S showed inhibited responses to collagen, ADP-fibrinogen and thrombin (Figs. 1a–1c). The extent of inhibition depended on the agent's concentration and increased after 60 min incubation. The control experiments showed that: (1) the 60 min incubation of platelets in the suspension buffer did not cause any cell aggregation, nor the significant decrease in the extent of cell response to collagen, ADP-fibrinogen and thrombin; and (2) the 60 min incubation of platelets in ganodermic acid S did not result in any cell aggregation, nor leaking of lactate dehydrogenase, Mg^{2+} or $[^{32}P]ATP$. Hence, the insertion of ganodermic acid S into the platelet membrane perturbed membrane function. The potency of the ganodermic acid S inhibition on platelet function in

response to inducers was ADP-fibrinogen $>$ collagen $>$ thrombin. For example, 50 % inhibition on platelet response to ADP-fibrinogen was found at $6 \mu M$ -ganodermic acid S for 10 min (Fig. 1a), that on collagen response appeared at $12.5 \mu M$ for 10 min (Fig. 1b), and that on thrombin response occurred at an higher concentration ($> 12.5 \mu M$) for a longer incubation period (> 20 min) (Fig. 1c).

The time-dependent inhibition of ganodermic acid S on platelet response to thrombin was further compared in respect of: (1) the cell aggregation; (2) the change in $[Ca^{2+}]_i$; and (3) phosphorylation of both 47 and 20 kDa proteins. Fig. 2 shows the results from a 60 min incubation of platelets at $12.5 \mu M$ -ganodermic acid S. The inhibition of cell aggregation was comparable with that of the change in $[Ca^{2+}]_i$, as well as with those of protein phosphorylation. Further observation showed that platelets in ganodermic acid S could fully respond to phorbol ester as a control even at 60 min incubation (results not shown). The time-dependent inhibition of ganodermic acid S on platelet response to thrombin might be due to a perturbation, by the agent, of membrane message transduction. Hence this kind of time-dependent effect on thrombin-stimulated phosphoinositide turnover was further explored.

Effect of ganodermic acid S on both resting and thrombin-stimulated platelet phosphoinositide metabolism

Previously, a 2 min time-course study of the effect of ganodermic acid S on platelet phosphoinositide metabolism indicated that the infiltration of ganodermic acid S into platelet membrane results in an immediate elevation of $[^{32}P]$ phosphatidylinositol ($[^{32}P]PI$), followed by the hydrolysis of each kind of phosphoinositide [3]. In a 60 min incubation there was no significant difference from control in the rate of ^{32}P incorporation into phosphatidylcholine (PC) (results not shown), and the time-course profiles of phosphoinositides and PA were different from those of controls. The 60 min profiles of phosphoinositides showed that, after the initial ganodermic acid S perturbation of phosphoinositide metabolism, the radioactivities of $[^{32}P]PI$ and $[^{32}P]$ phosphatidylinositol 4,5-bisphosphate ($[^{32}P]PIP_2$) levelled off at 2 min, and that of $[^{32}P]PIP$ levelled off after 10 min. In the case of incorporation of ^{32}P into PA, platelets at $> 10 \mu M$ -ganodermic acid S displayed an increase in $[^{32}P]PA$ by 6-fold that of control at 20 min incubation (Fig. 3).

The mass of both PI and PIP decreased and levelled off after 20 min incubation, whereas that of PIP_2 decreased initially and gradually gained back to the control level after 30 min incubation (Table 1). The specific radioactivity of PI did not increase with time, suggesting that the agent inhibited the non-equilibrium ^{32}P incorporation of PI that appeared in the control case. The result also showed that various concentrations of ganodermic acid S caused differential effects on phosphoinositide metabolism. Specifically, these are as follows. (1) Platelets in $6 \mu M$ -ganodermic acid S showed a 20 % decrease in PIP mass, but no significant change in either PI and PIP_2 , or in $[^{32}P]PA$. The hydrolysis of PIP might occur in this case. (2) Platelets in $12.5 \mu M$ -ganodermic acid S showed that the mass of PI, phosphatidylinositol 4-phosphate (PIP) and PIP_2 decreased by 10, 20 % and 15 % respectively, whereas $[^{32}P]PA$ increased to 6.5-fold the control value. This suggests that the hydrolysis of PIP_2 turned on the diacylglycerol (DG) formation pathway via $PI \rightarrow PIP \rightarrow PIP_2 \rightarrow DG$. After 20 min incubation the cells showed an additional 5 % decrease in PI, with an increase in PIP_2 to the control level. However, the radioactivity in $[^{32}P]PIP_2$ showed no change. In addition, after 30 min, $[^{32}P]PA$ gradually decreased. This kind of decrease appeared to be greater in platelets in $16 \mu M$ -ganodermic acid S. It may mean that the agent is involved in the activation of PA:CTP cytidyltransferase and/or

Fig. 2. Time-dependent inhibition by ganodermic acid S of thrombin-activated platelets in the changes in $[Ca^{2+}]_i$ ($\square$), phosphorylation of 20 ($\blacktriangle$) and 47 ($\blacktriangledown$) kDa proteins and aggregation ($\circ$)

Either gel-filtered or ^{32}P -labelled platelets (3×10^8 cells/ml) were preincubated in $12.5 \mu M$ -ganodermic acid S for a certain period, and then thrombin (0.1 unit/ml) was added for 1 min. Details of the experiments are given in the Experimental section. For each kind of experiment 100 % was the value obtained from the control under thrombin stimulation. All data are expressed as means $\pm$ S.E.M. ($n = 6$).

Fig. 3. Time-course effect of ganodermic acid S [GAS] on the incorporation of $[^{32}\text{P}]\text{PI}$ into $[^{32}\text{P}]\text{PI}$ (a), $[^{32}\text{P}]\text{PIP}$ (b), $[^{32}\text{P}]\text{PIP}_2$ (c) and $[^{32}\text{P}]\text{PA}$ (d)

^{32}P -labelled platelets (3×10^8 cells/ml) were added to ganodermic acid S at the concentrations indicated in the Figure. After various periods of incubation the radioactivity of each phospholipid class was estimated as detailed in the Experimental section. The circle (○) represents the control. All data are expressed as means $\pm$ S.E.M. ($n = 6$).

Table 1. Effect of ganodermic acid S (GAS) on the mass of phosphoinositides in platelets after various incubation times

Results (means $\pm$ S.E.M.) were obtained from seven separate experiments. The significance of the differences between the data obtained at 0 min and those after a certain incubation period was assessed by a paired t -test (* $P < 0.05$; ** $P < 0.01$).

Lipid class	GAS (μM)	Incubation time (min) ...	Mass (nmol/ 10^9 cells)					
			0	12	24	36	48	60
PIP_2	0		1.25 ± 0.09	1.23 ± 0.07	1.20 ± 0.10	1.13 ± 0.09	1.25 ± 0.11	1.21 ± 0.06
	6.25		1.26 ± 0.11	1.21 ± 0.04	1.21 ± 0.08	1.22 ± 0.12	1.17 ± 0.06	1.23 ± 0.06
	12.5		1.28 ± 0.12	$1.08 \pm 0.10^{**}$	$1.08 \pm 0.03^*$	$1.21 \pm 0.11^*$	1.25 ± 0.15	1.21 ± 0.16
	16		1.27 ± 0.14	$1.04 \pm 0.10^*$	$0.98 \pm 0.05^*$	$0.98 \pm 0.09^{**}$	1.07 ± 0.02	1.24 ± 0.08
PIP	0		1.97 ± 0.12	1.89 ± 0.10	1.88 ± 0.15	1.80 ± 0.13	1.87 ± 0.17	1.98 ± 0.18
	6.25		1.90 ± 0.13	$1.47 \pm 0.09^*$	$1.46 \pm 0.11^*$	$1.39 \pm 0.14^*$	$1.37 \pm 0.8^*$	$1.42 \pm 0.12^*$
	12.5		1.92 ± 0.20	$1.61 \pm 0.18^*$	$1.41 \pm 0.17^{**}$	$1.48 \pm 0.10^{**}$	$1.40 \pm 0.16^{**}$	$1.47 \pm 0.13^{**}$
	16		1.94 ± 0.14	1.71 ± 0.16	1.66 ± 0.15	1.76 ± 0.20	1.81 ± 0.19	1.71 ± 0.16
PI	0		11.06 ± 0.63	11.27 ± 0.86	11.25 ± 0.40	11.24 ± 0.63	11.50 ± 0.51	10.87 ± 0.79
	6.25		11.21 ± 0.67	11.04 ± 1.07	10.94 ± 0.84	10.82 ± 0.77	10.82 ± 0.74	10.80 ± 0.93
	12.5		11.27 ± 0.84	10.79 ± 0.93	$10.12 \pm 0.57^{**}$	$10.03 \pm 0.72^{**}$	$9.46 \pm 0.69^{**}$	$9.50 \pm 0.97^{**}$
	16		11.32 ± 0.95	$9.99 \pm 0.65^*$	$9.42 \pm 0.83^{**}$	$9.52 \pm 0.87^{**}$	$9.42 \pm 0.63^{**}$	$9.21 \pm 0.67^*$

Fig. 4. Time-dependent effect of ganodermic acid S on the thrombin-activated changes in human platelet [³²P]PI (a), [³²P]PIP (b), [³²P]PIP₂ (c) and [³²P]PA (d).

Bovine thrombin (0.1 unit/ml) was added to ³²P-labelled platelets (3 × 10⁸ cells/ml), which were preincubated at 12.5 μM-ganodermic acid S for the period indicated on the graph. The time on the abscissa refers to the incubation with thrombin. Estimation of the radioactivity of each phospholipid class was given in the Experimental section. The open circle (○) represents the control, which is equivalent to the 0 min incubation with ganodermic acid S. All data are expressed as means ± S.E.M. (n = 6).

PA hydrolysis. (3) Platelets in 16 μM-ganodermic acid S showed that the change in the mass of phosphoinositides was different from those found in the above cases. The time-course profiles of ³²P incorporation into phosphoinositides were also different from those found in the above cases. Notably, PIP decreased only by 10%, whereas PI mass decreased by 20% and [³²P]PA increased by 7-fold. This may mean that the rate of PIP synthesis from PI was higher than that of PIP hydrolysis occurring at 16 μM-ganodermic acid S. Furthermore, the increase of PIP₂ mass appeared after 30 min incubation with a lower rate than that found at 12.5 μM-ganodermic acid S. The radioactivity of [³²P]PIP₂, however, decreased continuously. This implies that another PI pool might be involved in PIP₂ resynthesis. In summary, the results showed that the agent initially induced the hydrolysis of phosphoinositides and turned on the DG-formation pathway. A prolonged incubation, on the other hand, resulted in another kind of effect, namely the release of PIP₂ by hydrolysis and a decrease in [³²P]PA.

How does ganodermic acid S affect thrombin-stimulated phosphoinositide turnover? A 2 min time-course study of the

change in radioactivity of each kind of phosphoinositide and PA was performed (Fig. 4). In the control, platelets aged at various stages displayed the parallel 2 min time-course profiles, showing an initial hydrolysis of PIP₂ with the resynthesis of PIP₂ after 10 s incubation (results not shown). The aged platelets showed an increase in [³²P]PI with time. However, under thrombin stimulation, the cells at various incubation times showed similar changes in the percentage of [³²P]PI (results not shown). The addition of thrombin to the platelets preincubated with ganodermic acid S produced similar 2 min time-course profiles of the changes in phosphoinositides and PA. However, the change in the level of each kind of phospholipid diminished in the cells on prolonged incubation. Fig. 4 illustrates the time-dependent effect of 12.5 μM-ganodermic acid S on thrombin-stimulated phosphoinositide turnover in platelets. Table 2 summarizes the net changes in the radioactivity of each phospholipid species obtained from Fig. 4. In Table 2 the change in PIP₂ is divided into two components: the decrease between the level at 0 and 10 s represents the hydrolysis of PIP₂, whereas the increase in the level from 10 to 60 s might represent the extent of PIP₂ resynthesis.

Table 2. Net change in the radioactivity of each phospholipid species of thrombin-activated platelets preincubated in ganodermic acid S for various periods

Results (mean $\pm$ S.E.M.) were summarized from those in Fig. 4. Significance of the differences between control platelets and those preincubated with ganodermic acid S was assessed by a paired *t* test (**P* < 0.05; ***P* < 0.01). Gel-filtered platelets were incubated at 12.5 μ M-ganodermic acid S. Net change in the radioactivity of each phospholipid class was obtained from the 2-min time-course study in Fig. 4. In parentheses are the time points at which the radioactivity was measured, and the difference represents either an increase or a decrease in the level.

Incubation period (min)	Phospholipid class ...	Net change in radioactivity (c.p.m./ 3×10^8 cells)				
		PI	PIP	PIP ₂		PA
		Decrease (0–60 s)	Increase (0–60 s)	Decrease (0–10 s)	Increase (10–60 s)	Increase (0–60 s)
0		4300 $\pm$ 780	5376 $\pm$ 728	1710 $\pm$ 357	5220 $\pm$ 558	5760 $\pm$ 161
10		5104 $\pm$ 606	5696 $\pm$ 542	1440 $\pm$ 414**	3330 $\pm$ 697*	3330 $\pm$ 291**
30		3712 $\pm$ 480	4672 $\pm$ 609	1440 $\pm$ 614	1800 $\pm$ 510*	1740 $\pm$ 219**
60		3248 $\pm$ 621	2752 $\pm$ 645*	360 $\pm$ 87**	360 $\pm$ 177**	780 $\pm$ 184*

Fig. 5. Scanning electron micrographs of human gel-filtered platelets after prolonged incubation in ganodermic acid S (magnification 6300 $\times$)

Gel-filtered platelets were preincubated at 37 °C for 30 min (a), and then treated with 12.5 μ M-ganodermic acid S for 20 min (b); 30 min (c); 40 min (d); 50 min (e); and 60 min (f). The asterisk (*) indicates the cell in a potato-like shape. Details are given in the Experimental section.

Fig. 6. Scanning electron micrographs of thrombin-activated human platelets preincubated in ganodermic acid S for various periods (magnification $6300\times$)

Thrombin (0.1 unit/ml) was incubated for 1 min with gel-filtered platelets (a) and with the platelets preincubated in $12.5\ \mu\text{M}$ -ganodermic acid S for 20 min (b), 30 min (c), 40 min (d), 50 min (e) and 60 min (f). The asterisk (*) indicates the cell in a spongy-sphere shape. The white arrow in (e) indicates a puffy discoid cell. Sample preparations were detailed in the Experimental section.

The preincubation of platelets in ganodermic acid S for 10, 30 and 60 min resulted in the inhibition of the resynthesis of PIP_2 by 40, 60 and 95 % of control respectively. A comparable result was also found with PA. However, decreases in PIP and PIP_2 became significant after 30 min incubation. Hence the inhibition of the formation of both PIP_2 and PA might be the primary effect of ganodermic acid S on thrombin-stimulated phosphoinositide interconversion.

Morphological changes in platelets at a prolonged incubation in ganodermic acid S

There was no significant change in platelet $[\text{Ca}^{2+}]_i$ during a prolonged incubation (60 min) at below $20\ \mu\text{M}$ -ganodermic acid S (results not shown). A morphological study by scanning electron microscopy was employed to see: (1) how platelets behave during a prolonged incubation in ganodermic acid S (Fig. 5); and (2) how the cells aged at different stages changed their morphology under thrombin stimulation (Fig. 6). A prolonged

incubation of platelets in ganodermic acid S resulted in time-dependent morphological change. Specifically, Fig. 5 shows this kind of change of platelets at $12.5\ \mu\text{M}$ -ganodermic acid S. Upon the addition of ganodermic acid S, the cells became discus-shaped with the protrusion of filopodia (width $< 200\ \text{nm}$) from the cell surface, the so-called 'spiculate discoid shape'. The cells kept this shape throughout 20 min incubation (Fig. 5b). Then, the filopodia gradually shortened and disappeared from the cell surface, and the cells became discoid-shaped (Figs. 5c and 5d). On 40 min incubation, 60 % of the cell population changed back to the discoid shape (Fig. 5d). At 60 min, 25 % of the cells swelled to a 'potato-like' shape (Fig. 5f). It is conceivable that this time-dependent change in platelet morphology is due to a gradual change in the distribution of ganodermic acid S between the membrane bilayer.

Under thrombin stimulation, the cells at different incubation stages showed a higher percentage of non-responding cells in the population after longer incubation periods. Specifically, Fig. 6

shows the morphological changes in thrombin-stimulated platelets preincubated at 12.5 μM -ganodermic acid S for various periods. Gel-filtered platelets stimulated by thrombin all became irregular, with a protrusion of pseudopods (width > 200 nm) (Fig. 6a). Platelets preincubated with ganodermic acid S for 20 min all responded to thrombin by rounding up to a spiculate irregular form (Fig. 6b). However, the addition of thrombin to platelets after prolonged incubation with ganodermic acid S for 30, 40, 50 and 60 min resulted in the non-responding cells forming 10, 18, 36 and 43 % of the population respectively. The non-responding cells did not have the irregular morphology seen in Fig. 6(a), but appeared either as puffy discoids or as spongy-like spheres. The latter shape represents cells lysed by the synergistic effect of thrombin and ganodermic acid S. The observation confirmed that a prolonged incubation of platelets in ganodermic acid S caused the time-dependent inhibition of platelet response to thrombin (Fig. 2).

DISCUSSION

Human platelets behave differently at various concentrations of ganodermic acid S. The cells aggregate at above 20 μM -agent, owing to the activation of PIP_2 hydrolysis as well as the onset of phosphoinositide interconversion [3]. A different part of the present study demonstrated that, at below 20 μM -ganodermic acid S, platelets display inhibited responses to ADP-fibrinogen, collagen and thrombin (Fig. 1). The inhibition was not due to the denaturation of these three agonists by the agent, since the inhibitions are enhanced at prolonged incubation. The inhibitory potency of ganodermic acid S differs among platelet responses to these agonists, indicating different mechanisms of action of these agonists on the platelet membrane [9,10,16–27]. Among these three kinds of platelet response, the integrity of platelet membrane structure is more important to the stimulations by ADP-fibrinogen and collagen than to that by thrombin. A similar finding is obtained with platelets in various kinds of detergents, where perturbation of platelet membrane structure results in inhibited responses to both ADP-fibrinogen and collagen, but not to thrombin [4].

The infiltration of ganodermic acid S into platelet membrane results in a biphasic effect on phosphoinositide metabolism (Table 1 and Fig. 3). The initial-phase effect involves the hydrolysis of PIP and PIP_2 . The former is effective at 6 μM -ganodermic acid S; the latter occurs at > 10 μM , showing DG formation via $\text{PI} \rightarrow \text{PIP} \rightarrow \text{PIP}_2 \rightarrow \text{DG}$. The second-phase effect occurs after 30 min incubation of the cells in ganodermic acid S. It involves PIP_2 resynthesis and a decrease in $[\text{P}^{32}\text{P}]$ PA. Platelets at this stage also show the inhibited response to thrombin in that the PIP_2 resynthesis in phosphoinositide turnover is inhibited (Table 2 and Fig. 4c). A decrease in the level of $[\text{P}^{32}\text{P}]$ PA indicates that the agent may also cause the activation of PA:CTP cytidyltransferase and/or PA hydrolysis. In response to thrombin, platelets incubated in ganodermic acid S for various periods show the same degree of decrease in $[\text{P}^{32}\text{P}]$ PI (Fig. 4a), even though the PI pool size is reduced by 20 %. Hence, a thrombin-sensitive pool exists in platelet membrane, as suggested by other workers [11,16,28]. How can ganodermic acid S trigger the biphasic effect on platelet phosphoinositide metabolism? One of the possible explanations may be deduced from the morphological study by scanning electron microscopy (Figs. 5 and 6).

The scanning-electron-microscopic study shows that platelets display a sequential time-dependent morphological change in ganodermic acid S (Fig. 5). The cell morphology is governed by both membrane morphology and cytoskeleton [5,6,29,30]. The former includes both the lipid bilayer and the membrane skeleton. Since platelets in ganodermic acid S do not show a change in

$[\text{Ca}^{2+}]_i$, the morphological change found in this study may be due to the effect of ganodermic acid S on platelet membrane morphology. It has been suggested that a time-course study of the effect of a membrane-acting agent on platelet morphology may reflect the 'Flip Flop' phenomenon of the agent in the platelet membrane bilayer [4]. Also, the bilayer-couple hypothesis has been used to explain the effect of membrane-acting agents on platelet membrane morphology [5]. The platelet spicules are due to an accumulation of solute in the membrane outer leaflet, whereas a spherical platelet is due to concentrating of the solute in the inner leaflet. In this study the time-dependent morphological change in platelets in ganodermic acid S may reflect a gradual change in the distribution of ganodermic acid S between the two membrane leaflets and the Flip Flop phenomenon. It is conceivable that ganodermic acid S initially inserts into the platelet membrane outer leaflet, giving rise to the spiculate discoid shape of the platelets (Fig. 5b). Owing to a slow Flip Flop rate of ganodermic acid S movement between the membrane bilayer, it takes over 20 min for platelets to become discoid shape; these platelets then swell up to a potato-like shape (Figs. 5c–5f). The morphology in the latter case implies that the agent concentrates more in the inner leaflet than in the outer one. Another scanning-electron-microscope study confirms that platelets aged in ganodermic acid S respond to thrombin with an increase in the percentage of the non-responding cells (Fig. 6). This observation is comparable with: (1) the aggregation study (Figs. 1c and 2); (2) the drug effect on phosphoinositide metabolism (Table 1; Fig. 3); and (3) the study of ganodermic acid S inhibition of thrombin-stimulated phosphoinositide turnover (Fig. 4). It is conceivable that the accumulation of ganodermic acid S in the different membrane leaflets causes the biphasic effect on phosphoinositide metabolism.

The current study and a previous one [3] indicate that the insertion of ganodermic acid S into the platelet membrane brings about multiple effects on platelet phosphoinositide metabolism. The differential effects may depend on the differential accumulation of the agent in the two membrane leaflets.

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