Sphingosine enhances platelet aggregation through an increase in phospholipase C activity by a protein kinase C-independent mechanism

Tsutomu HASHIZUME, Takashi SATO* and Tatsuzo FUJII

Department of Biochemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan

Sphingosine (a potent inhibitor of protein kinase C) at 5–10 μM , which are concentrations lower than those that inhibit this enzyme activity, enhanced the aggregation of rabbit platelets induced by low concentrations of U46619, platelet-activating factor, thrombin and arachidonic acid, whereas H-7 and staurosporine, other protein kinase C inhibitors, failed to do so. Of the sphingosine analogues which also inhibit protein kinase C, psychosine and lyso- $\text{G}_{\text{M}3}$ did not show such an enhancing effect. Sphingosine promoted both $\text{Ins}(1,4,5)\text{P}_3$ formation and an increase in the cytoplasmic free Ca^{2+} concentration in response to all the agonists used. Furthermore, the hydrolytic action of exogenously added phospholipase C (from *Clostridium perfringens*) on platelet membrane phospholipids was dose-dependently enhanced by pretreatment of the platelets with sphingosine. These results imply that sphingosine, at relatively low concentrations, brings about hyperaggregability of the platelets by the agonists employed, probably owing to enhancement of the phospholipase C activity. Such an effect appears to be induced by a mechanism independent of protein kinase C inhibition. We suggest that sphingosine might act as a positive modulator for the stimulus–response coupling in the platelets.

INTRODUCTION

Long-chain sphingoid bases, such as sphingosine, have been shown to inhibit protein kinase C [1–4] and to suppress cellular responses: aggregation, secretion and intracellular alkalinization in platelets [5–7]; the oxidative burst in neutrophils [8]; and neurite outgrowth in PC12 cells [9]. Such effects of sphingosine on cellular functions were suggested to be due to its inhibitory action on protein kinase C. However, the effects of sphingosine are not limited to those on protein kinase C. Sphingosine was shown to have the ability to inhibit Ca^{2+} /calmodulin-dependent protein kinase of GH_3 pituitary cells [10], Na^+/K^+ -ATPase purified from rat brain synaptosomes [11] and thyrotropin-releasing hormone binding to pituitary cells [12]. On the contrary, it has been reported that sphingosine and related derivatives activate tyrosine kinase of the epidermal-growth-factor receptor in human WI-38 fetal lung fibroblasts or A431 epidermoid carcinoma cells [13–15], stimulate the cellular proliferation of Swiss 3T3 cells through a protein kinase C-independent pathway [16], and mediate intracellular Ca^{2+} release in a smooth-muscle cell line [17]. One could thus expect that sphingosine activates certain cellular responses, other than suppressing those via inhibition of protein kinase C.

We evaluated in this work an effect of sphingosine on platelet aggregation induced by some agonists, e.g. U46619 (a thromboxane mimetic analogue), platelet-activating factor (PAF), thrombin and arachidonic acid, as compared with that of other inhibitors of protein kinase C. These agonists are known to induce the phospholipase C (PLC) activation responsible for platelet aggregation [18]. This PLC activation results in the formation of 1,2-diacylglycerol, which activates protein kinase C, and $\text{Ins}(1,4,5)\text{P}_3$, which induces elevation of the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). We therefore also examined the influence of sphingosine on PLC activation in rabbit platelets by measuring the $\text{Ins}(1,4,5)\text{P}_3$ formation and the increase in $[\text{Ca}^{2+}]_i$,

to obtain further evidence as to the mechanism of action of sphingosine on platelet aggregation.

EXPERIMENTAL

Materials

Sphingosine (from bovine brain sphingomyelin), psychosine (galactosylsphingosine), PAF, arachidonic acid and PLC (from *Clostridium perfringens*) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sphingosine was dissolved in ethanol at a concentration 300-fold higher than the final concentration (final ethanol concn. 0.33%). U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy-prostaglandin $\text{F}_{2\alpha}$) was obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Thrombin (bovine plasma) was from Mochida Pharmaceutical Co. (Tokyo, Japan). [^3H]Arachidonic acid (76 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Fura2 penta-acetoxy-methyl ester (Fura2-AM) was from Dohjin Chemical Co. (Kumamoto, Japan). BSA (fraction V) was from Boehringer (Mannheim, Germany). Staurosporine and H-7 were from Kyowa Medex (Tokyo, Japan) and Seikagaku Kogyo Co. (Tokyo, Japan) respectively. Lyso- $\text{G}_{\text{M}3}$ and *p*-bromophenacyl bromide (*p*-BPB) were from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were obtained from commercial sources.

Preparation of platelets

Fresh rabbit blood anti-coagulated with 0.1 vol. of 1% EDTA was centrifuged at 230 *g* for 10 min at room temperature to obtain platelet-rich plasma. The platelets separated from the platelet-rich plasma were washed twice as described previously [19]. Finally, the platelet suspension was adjusted to 5×10^8 cells/ml in modified Tyrode–Hepes buffer (137 mM-NaCl, 2.7 mM-KCl, 1 mM- MgCl_2 , 5.6 mM-glucose, 2.9 mM- NaH_2PO_4 , 3.8 mM-Hepes, pH 7.35) containing 0.35% BSA.

Abbreviations used: PLC, phospholipase C; PLA_2 , phospholipase A_2 ; $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; *p*-BPB, *p*-bromophenacyl bromide; PAF, platelet-activating factor.

* To whom correspondence should be addressed.

Platelet aggregation

Platelet aggregation was measured as the change in light transmission at 37 °C in the presence of 1 mM-CaCl₂, with an aggregometer (NKK Hema Tracer 1; Niko Bioscience Co., Tokyo, Japan).

Ins(1,4,5)P₃ formation

Washed platelets, pretreated with various concentrations of sphingosine at 37 °C for 1 min in the presence of 1 mM-CaCl₂, were exposed to each agonist for 15 s. After the reactions had been terminated by addition of 0.2 vol of 20% (v/v) HClO₄, the mixtures were centrifuged at 2000 *g* for 15 min at 4 °C. The supernatants obtained were adjusted to pH 7.5 with KOH and then centrifuged as described above to remove the precipitated KClO₄. The amount of Ins(1,4,5)P₃ in the supernatant was measured with an Ins(1,4,5)P₃ assay system (Amersham International, Amersham, Bucks., U.K.).

Measurement of [Ca²⁺]_i

The loading of platelets with a fluorescent dye, Fura2, was essentially carried out by the method of Pollock *et al.* [20]. Briefly, washed platelets were incubated with 2 μM-Fura2-AM at 37 °C for 30 min and then washed once as described above. The Fura2-loaded platelets (5 × 10⁸ cells/ml) were treated at 37 °C for 1 min with various concentrations of sphingosine in the presence of 1 mM-CaCl₂, and then diluted to 1.5 × 10⁸ cells/ml with Tyrode-Hepes buffer containing 1 mM-CaCl₂. Fluorescence from the suspension was continuously monitored with a spectrofluorimeter (F-2000; Hitachi), with excitation at 340 nm and 380 nm and emission at 500 nm. After each experiment, the cells were lysed with 0.1% Triton X-100 to obtain the maximum fluorescence. Thereafter, the minimum fluorescence was measured after addition of 5 mM-EGTA and Tris base (pH of the lysed cells being adjusted to 8.5). [Ca²⁺]_i was then calculated by the method of Grynkiewicz *et al.* [21].

Activity of exogenously added PLC

The platelet-rich plasma was incubated with [³H]arachidonic acid (2 μCi/ml) at 37 °C for 1 h and then washed as described above to obtain [³H]arachidonic acid-labelled platelets. The platelets were pretreated with 50 μM-*p*-BPB, in order to inactivate the endogenous phospholipase A₂ (PLA₂) and PLC, and then washed to remove the excess drug. The platelets were treated at 37 °C for 1 min with or without sphingosine in the presence of 1 mM-CaCl₂, and further with PLC (*C. perfringens*). After 2 min, the reaction was terminated by addition of chloroform/methanol/conc. HCl (200:200:1, by vol.) and then diacylglycerol formation, as an indicator of the enzyme activity, was analysed as described previously [22].

RESULTS

Effect of sphingosine on platelet aggregation

The effect of sphingosine on platelet aggregation induced by U46619 is shown in Figs. 1 and 2(a). When platelets were preincubated with various concentrations of sphingosine and then exposed to a high concentration of U46619 (2 μM), which must induce full aggregation, sphingosine dose-dependently inhibited the aggregation at concentrations of 20 μM and higher. On the other hand, sphingosine at low concentrations in the range 2.5–15 μM significantly enhanced the aggregation under stimulation with a low concentration of U46619 (0.3 μM), which by itself induces only slight aggregation. Such an enhancing effect was biphasic: up to 7.5 μM-sphingosine the effect increased with the concentration, but at higher concentrations it tended to

decrease gradually, and at higher than 20 μM-sphingosine rather exerted a suppressive effect. Even when the platelets were pretreated with indomethacin (10 μM) or BW755C (an inhibitor of both cyclo-oxygenase and lipoxygenase; 50 μM), no effect was observed on sphingosine-induced hyperaggregability (results not shown). In contrast, treatment of platelets with H-7 or staurosporine, known to be protein kinase C inhibitors [23,24], did not have any enhancing effect on the aggregation (Fig. 2b).

Likewise, sphingosine had a potentiating effect on the aggregation induced by a low concentration of PAF, thrombin or arachidonic acid (Fig. 3), whereas H-7 or staurosporine did not (results not shown). The mode of potentiation under stimulation with PAF was expressed as the interference with the dissociation of aggregation, rather than as the increase in the extent of maximum aggregation (Fig. 3). These potentiations were not

Fig. 1. Effect of sphingosine on platelet aggregation induced by U46619

Washed platelets were incubated at 37 °C for 1 min with various concentrations of sphingosine and then exposed to (●) 0.3 μM- or (▲) 2 μM-U46619 in an aggregometer. The change in light transmission of the suspension after 5 min stimulation with 2 μM-U46619 in the absence of sphingosine was taken as 100%. Each point represents the average of duplicate determinations. Similar results were obtained in two other experiments.

Fig. 2. Effects of sphingosine, H-7 and staurosporine on U46619-induced platelet aggregation

Washed platelets were incubated at 37 °C with various concentrations of sphingosine for 1 min (a), with 100 μM-H-7 for 5 min or 100 nM-staurosporine for 1 min (b), and then exposed to 0.3 μM-U46619. Each trace is the typical result of two to four separate experiments.

Fig. 3. Effect of sphingosine on platelet aggregation induced by PAF, thrombin or arachidonic acid

Washed platelets were incubated at 37 °C for 1 min with 10 μ M-sphingosine (Sph.) or the ethanol vehicle (None), and then exposed to 1 nM-PAF (a), 0.008 unit of thrombin/ml (b) or 20 μ M-arachidonic acid (c). Each trace is the typical result of three separate experiments.

Fig. 4. Effect of sphingosine on Ins(1,4,5) P_3 formation induced by U46619

Washed platelets were incubated at 37 °C for 1 min with various concentrations of sphingosine in the presence of 1 mM- CaCl_2 , and then stimulated with 0.5 μ M-U46619 (●) or the buffer vehicle (○) for 15 s. Ins(1,4,5) P_3 formed was determined as described in the Experimental section. Each point represents the average of duplicate determinations. Similar results were obtained in two other experiments.

affected by indomethacin or BW755C, except for arachidonic acid stimulation (results not shown). The evidence that sphingosine enhanced arachidonic acid-induced aggregation indicates that it can potentiate a stimulus-response sequence via physiologically generated thromboxane A_2 .

To compare the structural requirements of sphingosine for potentiating platelet aggregation with those for protein kinase C inhibition previously reported by others [3], two sphingosine analogues, psychosine (galactosylsphingosine) and lyso- G_{M3} , were examined. Neither, however, caused any potentiation at 1–20 μ M of U46619-induced aggregation, although they exhibited an inhibitory effect at more than 20 μ M (results not shown).

Effect of sphingosine on Ins(1,4,5) P_3 formation

Since PLC activation following U46619 binding to the receptor is known to play an essential role in mediating U46619-induced aggregation, the effect of sphingosine on PLC activation was examined in order to elucidate the mechanism by which the

Table 1. Effect of sphingosine on Ins(1,4,5) P_3 formation and the increase in $[\text{Ca}^{2+}]_i$ stimulated by PAF, thrombin or arachidonic acid

Washed platelets were treated with 10 μ M-sphingosine (Sph.) or the ethanol vehicle at 37 °C for 1 min in the presence of 1 mM- CaCl_2 and then stimulated for 15 s with 5 nM-PAF, 0.02 unit of thrombin/ml or 100 μ M-arachidonic acid. Ins(1,4,5) P_3 formed was determined as described in the Experimental section. For measurement of $[\text{Ca}^{2+}]_i$, Fura2-loaded platelets were treated with or without sphingosine as described above, and then the change in $[\text{Ca}^{2+}]_i$ induced by each agonist was determined as described in the Experimental section. The results are the means $\pm$ s.d. of triplicate determinations. Similar results were obtained in another experiment. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, **** $P < 0.001$, compared with each vehicle.

Additions	Ins(1,4,5) P_3 (pmol/ 10^9 cells)	$[\text{Ca}^{2+}]_i$ (nM)
Vehicle + none	7.1 $\pm$ 0.5	84 $\pm$ 9
Sph. + none	7.0 $\pm$ 1.5	88 $\pm$ 6
Vehicle + PAF	11.9 $\pm$ 0.5	399 $\pm$ 23
Sph. + PAF	15.1 $\pm$ 1.8*	554 $\pm$ 15****
Vehicle + thrombin	10.3 $\pm$ 1.2	392 $\pm$ 14
Sph. + thrombin	12.7 $\pm$ 0.5**	566 $\pm$ 18****
Vehicle + arachidonic acid	9.4 $\pm$ 1.6	289 $\pm$ 24
Sph. + arachidonic acid	12.8 $\pm$ 0.5***	518 $\pm$ 46****

aggregation was accelerated. As shown in Fig. 4, U46619-induced Ins(1,4,5) P_3 formation was enhanced with an increase in sphingosine concentration up to 10 μ M. At this concentration, sphingosine increased the formation about 3.1-fold compared with that in the absence of sphingosine. Sphingosine alone, i.e. without the added agonist, had no effect up to 30 μ M.

To evaluate further the acceleration of PLC by sphingosine, its potentiation of Ins(1,4,5) P_3 formation in response to other agonists which can induce PLC activation in a stimulus-response sequence was examined. The results in Table 1 clearly show that 10 μ M-sphingosine caused significant enhancement of Ins(1,4,5) P_3 formation induced by PAF, thrombin and arachidonic acid.

Effect of sphingosine on the increase in $[\text{Ca}^{2+}]_i$

Measurement of the Fura2 signal is mostly performed with low concentrations of cells, to prevent interference with the fluorescence by the turbidity of the cell suspension. For this reason, after platelets had been pretreated with sphingosine under the same conditions as in the other experiments, the suspension was diluted with buffer and then subjected to the measurement. Fig. 5 shows a typical result, indicating that the increase in $[\text{Ca}^{2+}]_i$ caused by U46619 was accelerated by treatment with sphingosine (5–15 μ M). The magnitude of the acceleration was about 2.7-fold with 10 μ M-sphingosine, being compatible with the enhancement of Ins(1,4,5) P_3 formation, as shown in Fig. 4. Similarly, significant potentiation of the increase in $[\text{Ca}^{2+}]_i$ by sphingosine was also obtained in platelets exposed to PAF, thrombin or arachidonic acid, as seen in Table 1.

Effect of sphingosine on the activity of exogenously added PLC

Our results indicate that sphingosine enhanced the endogenous PLC activation by the agonists used. To define the mechanism underlying the observed effect of sphingosine, we examined whether or not sphingosine affects the activity of exogenously added PLC from *C. perfringens* on platelet membranes as a substrate. It has been reported that the bacterial PLC caused platelet activation by stimulating the turnover of phosphoinositides [25], demonstrating that added PLC can exhibit a similar action to that of endogenous PLC. In order to depress the action of endogenous PLC, platelets were pretreated with *p*-BPB,

Fig. 5. Effect of sphingosine on the increase in $[Ca^{2+}]_i$ induced by U46619

Fura2-loaded platelets were incubated at 37 °C for 1 min with various concentrations of sphingosine (Sph.) and then, after dilution of the suspension to 1.5×10^8 cells/ml with buffer, 5 μ M-U46619 was added, as indicated by the arrow. Each trace shows $[Ca^{2+}]_i$ calculated from the fluorescence signal of Fura2 as described in the Experimental section. The traces shown are representative of three separate experiments.

Fig. 6. Effect of sphingosine on the action of exogenously added PLC on platelet membranes

$[^3H]$ Arachidonic acid-labelled platelets were pretreated with 50 μ M-*p*-BPB and then washed. (a) The platelets were incubated at 37 °C for 1 min with 10 μ M-sphingosine (●) or the ethanol vehicle (○) and then exposed to various concentrations of PLC for 2 min. (b) The platelets were incubated at 37 °C for 1 min with various concentrations of sphingosine and then exposed to 0.1 unit of PLC/ml (▲) or the buffer vehicle (△). $[^3H]$ Diacylglycerol formed was determined as described in the Experimental section. Each point represents the mean $\pm$ S.D. of triplicate determinations. Similar results were obtained in two other experiments.

which is known to inhibit the PLC at high concentrations, as well as PLA_2 [26,27].

As shown in Fig. 6(a), the exogenous PLC dose-dependently produced 1,2-diacylglycerol from membrane lipids of $[^3H]$ -arachidonic acid-labelled platelets. When the platelets were

pretreated with 10 μ M-sphingosine, the production was significantly enhanced with any concentration of the PLC used (0.05–0.2 unit/ml). Furthermore, 1,2-diacylglycerol formation in response to 0.1 unit of PLC/ml was increased by sphingosine in a concentration-dependent manner (Fig. 6b). Sphingosine alone did not induce any change in the 1,2-diacylglycerol production.

DISCUSSION

Since it was reported that sphingosine inhibits protein kinase C [1–4], much evidence has been presented showing various roles of sphingosine in the physiological processes in many cells (for reviews see [28–30]). In the present study, we found that sphingosine enhanced platelet aggregation induced by a low concentration of U46619, PAF, thrombin or arachidonic acid. Because the presence of indomethacin or BW755C did not abolish such an effect of sphingosine in response to U46619, PAF or thrombin, neither PLA_2 nor cyclo-oxygenase is supposed to be involved in such an effect. Moreover, these effects appear to be caused by a mechanism independent of protein kinase C inhibition, as revealed by the following evidence: (i) in contrast with sphingosine, other potent inhibitors of protein kinase C, H-7 and staurosporine [23,24], failed to enhance the aggregation, and (ii) the concentration of sphingosine enhancing the aggregation was 15 μ M or less, which is lower than the known concentration required to inhibit protein kinase C of platelets (25–100 μ M) [5,31].

Since psychosine and lyso- G_{M3} , which are structurally related to sphingosine, and are known to have activities similar to or stronger than those of sphingosine as inhibitors of protein kinase C [3], did not exert any potentiating effect on U46619-induced aggregation, the potentiating activity of sphingosine appears to be specific, unlike the inhibitory activity toward protein kinase C.

The activation of PLC which leads to the formation of 1,2-diacylglycerol, which activates protein kinase C [32], and of $Ins(1,4,5)P_3$, which releases Ca^{2+} from internal stores [33], is an essential step in platelet activation [18]. The finding here that sphingosine promoted the $Ins(1,4,5)P_3$ formation and the increase in $[Ca^{2+}]_i$ in response to the agonists used suggests that the acceleration of PLC activation by sphingosine is involved in the mechanism by which sphingosine potentiates platelet aggregation. It was recently reported that sphingosine potentiates diacylglycerol formation induced by thrombin through the removal of the negative-feedback regulation of protein kinase C in platelets [34]. However, our finding that the activity of PLC exogenously added to platelet membranes was also enhanced by sphingosine supports our idea that the effect is induced by a mechanism independent of protein kinase C. In addition, this result also implies that sphingosine may activate PLC through its direct interaction with the PLC molecule or by increasing the susceptibility of the enzyme to the substrate, probably owing to a certain change in the packing of the membrane phospholipids.

On the other hand, high concentrations (over 20 μ M) of sphingosine inhibited platelet aggregation. This phenomenon may be explained by the inhibitory action on protein kinase C or by the cytotoxic effect of sphingosine at high concentrations [35,36]. The decrease by high concentrations of sphingosine of the hyperaggregability of U46619-stimulated platelets also seems to be due to this effect.

If we assume that almost all the added sphingosine in the concentration range that enhances U46619-induced aggregation (5–10 μ M) is incorporated into platelets, the amount is estimated to be 13–26 pmol/ 10^6 cells under the conditions used here. In neutrophils and HL-60 cells, free sphingosine is reported to be present in the range of 2–12 pmol/ 10^6 cells [37,38]. Therefore the

amount of sphingosine that must be incorporated into the platelet membrane and contribute to induction of hyper-aggregability does not seem to be an amount that extremely exceeds the physiological content.

It is not clear yet whether or not sphingosine actually plays a role as a modulator in the signal-transduction system under stimulation. However, it was reported that, in cultured neuroblastoma cells and erythrocytes, the plasma membrane contains sphingomyelinase [39,40]. Slife *et al.* [41] suggested that free sphingosine, generated through a stimulated sphingomyelin cycle in liver plasma membranes, may regulate the stimulus-response coupling. Furthermore, Kolesnick [42] reported the suggestive evidence that 1,2-diacylglycerol stimulated sphingomyelin hydrolysis in pituitary cells. Thus there is a possibility that a certain stimulus in platelets might induce the generation of sphingosine, which contributes to the subsequent responses, although further studies are needed to clarify this.

In conclusion, we suggest that sphingosine enhanced platelet aggregation in response to a relatively low concentration of U46619, PAF, thrombin or arachidonic acid through its enhancing effect on the PLC activity. This effect appears to be induced by a mechanism independent of the inhibition of protein kinase C. We assume that sphingosine might play a positive role in the cellular signal transduction system, other than a negative effect on protein kinase C.

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