Biochem. J. (1992) 285, 745–751 (Printed in Great Britain)

Suppression by wortmannin of platelet responses to stimuli due to inhibition of pleckstrin phosphorylation

Yutaka YATOMI,*† Osamu HAZEKI,* Shoji KUME‡ and Michio UI*§
* Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, Japan, and ‡ Department of Clinical Laboratory Medicine, Yamanashi Medical College, Yamanashi, Japan

Studies were made of inhibition by wortmannin, a fungal metabolite, of human platelet responses to various stimuli. Wortmannin at concentrations as low as 1–100 nM inhibited several receptor-agonist-induced 5-hydroxytryptamine release from platelets, without affecting agonist-induced increases in the intracellular concentration of Ca^{2+}. Phorbol 12-myristate 13-acetate (PMA), an active tumour promoter, caused 5-hydroxytryptamine release when combined with a low concentration of ionomycin, and platelet aggregation by itself; these effects of the phorbol ester were also inhibited by wortmannin as well as by staurosporine, a potent, although non-specific, protein kinase C (PKC) inhibitor, in a similar molar concentration range. The platelet responses to the receptor agonists or PMA were accompanied by increased incorporation of $[^{32}P]P_i$ into pleckstrin, a protein selectively expressed in platelets and other blood cells arising from haematopoietic stem cells, as a result of PKC activation in the intact cells. The pleckstrin phosphorylation was inhibited by wortmannin in ways mostly similar to those in which it inhibited the 5-hydroxytryptamine-release responses. Nevertheless, wortmannin failed to inhibit PKC activity measurable in a cell-free asay system which is highly susceptible to staurosporine. Nor did it inhibit the translocation of cytosolic PKC to membranes induced by addition of PMA to platelet cells. Thus wortmannin, which is not a direct inhibitor of PKC, could interfere with the kinase-dependent phosphorylation of pleckstrin, which may play an important role in the cellular responses to receptor stimulation.

INTRODUCTION

Wortmannin is a mycotoxin produced by *Fusarium oxysporum* [1–3]. Administration of this toxin was reported to produce various toxic effects in animals [2,3]. A derivative of wortmannin was shown to interact with certain pathway(s) of signal transduction, thereby inhibiting the respiratory burst in neutrophils [4].

As this fungal metabolite induced severe haemorrhage in animals [2,3], it would be possible to assume that this agent exerts effects on physiological functions of platelets. We therefore studied effects of wortmannin on human platelets, a model system for stimulus–response coupling pathways that can be expected to have general implications for other cell types [5–7]. Unexpectedly, wortmannin inhibited platelet physiological responses to receptor agonists, probably as a result of interference with the phosphorylation of a 47 kDa protein, pleckstrin, a well-known protein kinase C (PKC) substrate expressed selectively in haematopoietic cell lineage [5,8–11], despite its apparent failure to affect the PKC activity in a cell-free system.

MATERIALS AND METHODS

Materials

Wortmannin, staurosporine and 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2) were kindly given by Sandoz (Basel, Switzerland), Kyowa Medex (Tokyo, Japan), and Ono Pharmaceutical Co. (Osaka, Japan) respectively. The following materials were purchased from the indicated suppliers: phorbol 12-myristate 13-acetate (PMA) (Pharmacia, Stockholm, Sweden); ionomycin (Calbiochem, La Jolla, CA, U.S.A.); thrombin (Green Cross, Osaka, Japan); H-7 (Seikagaku-Kogyo, Tokyo, Japan); sphingosine, aspirin, dibutyryl cyclic AMP, histone III-S, phosphatidylserine (Sigma, St. Louis, MO, U.S.A.); collagen (Hormon-Chemie, Munich, Germany); fibrinogen (Kabi, Stockholm, Sweden); fura2-AM (Molecular Probes, Eugene, OR, U.S.A.); 5-hydroxy[^{14}C]tryptamine (56.4 mCi/mmol), $[^{32}P]P_i$ (8500–9120 Ci/mmol), [^{3}H]phorbol 12,13-dibutyrate (PDBu) (19.1 Ci/mmol) (Du Pont–NEN, Boston, MA, U.S.A.); [γ-^{32}P]ATP (> 7000 Ci/mmol) (ICN Biomedicals, Costa Mesa, CA, U.S.A.).

Handling of wortmannin

Stock solutions (10 mM) of wortmannin were prepared in dimethyl sulphoxide and further diluted with incubation medium just before use. The final concentration of dimethyl sulphoxide was kept at 0.01%, which had been found to have no effect on platelet functions. Unless otherwise mentioned, the platelet suspension had been incubated with wortmannin for 5 min at 37 °C before the start of stimulation of the platelets. Essentially the same effect of wortmannin on the platelet 5-hydroxytryptamine-release reaction was observed when the preincubation time was changed from 1 to 10 min (results not shown). Wortmannin by itself did not stimulate platelets; there were no detectable changes in cell shape, 5-hydroxytryptamine or lactate dehydrogenase release, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or protein phosphorylation during incubation of platelets with wortmannin alone (results not shown).

Platelet preparation

Preparation of platelet-rich plasma and the procedure for washing platelets were described previously [12]. The final platelet pellet was suspended in Hepes–Tyrode's buffer (129 mM-NaCl, 8.9 mM-$NaHCO_3$, 2.8 mM-KCl, 0.8 mM-KH_2PO_4, 0.8 mM-$MgCl_2$,

Abbreviations used: PKC, protein kinase C; STA_2, 9,11-epithio-11,12-methano-thromboxane A_2; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

† Present address: Department of Clinical Laboratory Medicine, Yamanashi Medical College, Yamanashi, Japan.
§ To whom correspondence should be addressed.

1 mg of glucose/ml, 10 mM-Hepes, pH 7.4) at a cell density of 3×10^8/ml. The suspension was further supplemented with 1 mM-Ca^{2+} if not specified otherwise. All experiments using intact platelet suspensions were performed at 37 °C.

Measurements of 5-hydroxytryptamine release from platelets

Platelet-rich plasma was incubated with 5-hydroxy[^{14}C]tryptamine (100 nCi/ml) at 37 °C for 45 min. The platelets were then washed twice and resuspended in Hepes–Tyrode's buffer. This ^{14}C-labelled platelet preparation was incubated with stimulants for indicated lengths of time, and the release reaction was stopped by addition of 0.1% glutaraldehyde to the reaction mixture, quickly followed by immersing the mixture-containing tubes in an ice-cold bath. After centrifugation at 10000 *g* for 40 s at 4 °C, the radioactivity in the supernatant was determined by liquid-scintillation counting. Since collagen-induced 5-hydroxytryptamine release was dependent on aggregation (results not shown), as previously described [13,14], the reaction was observed under conditions in which platelets were aggregated, as described below. When indicated, platelet suspension was preincubated with 500 μM-aspirin, a cyclo-oxygenase inhibitor, for 15 min at 37 °C. This concentration of aspirin completely blocked 10 μM-arachidonate-induced platelet aggregation and 5-hydroxytryptamine release (results not shown). The results were expressed as the percentage release of the total 5-hydroxy[^{14}C]tryptamine radioactivity that had been incorporated into platelets. Initial experiments on the time course of thrombin-induced 5-hydroxy-[^{14}C]tryptamine release and on the effect of pretreating platelets with 1 μM-imipramine revealed that no significant re-uptake of 5-hydroxy[^{14}C]tryptamine by the platelets occurred over 5–15 min after the agonist addition. Therefore further experiments were conducted without pretreatment of the platelets with imipramine.

Platelet aggregation

Platelet aggregation was measured in a Platelet Ionized Calcium Aggregometer (Chrono-Log, Havertown, PA, U.S.A.). The instrument was calibrated with a platelet suspension for zero light transmission and Hepes–Tyrode's buffer for 100% transmission. Platelet suspensions were kept at 37 °C under continuous stirring at 1000 rev./min. Purified human fibrinogen (500 μg/ml) was added to platelet suspensions shortly before the addition of stimulants.

$[Ca^{2+}]_i$ measurement with the use of fura2

Platelet-rich plasma was incubated at 37 °C with 3 μM-fura2-AM for 30 min. The platelets were then washed twice and resuspended in Hepes–Tyrode's buffer. $[Ca^{2+}]_i$ indicated by fura2 signals was measured in a CAF-100 instrument (Japan Spectroscopic Co., Tokyo, Japan) with an excitation wavelength of 340/380 nm and emission at 500 nm. $[Ca^{2+}]_i$ values were calculated from fura2 ratios (R: the ratio of the fluorescence intensity at 340 nm to that at 380 nm) according to the equation [15]:

$$[Ca^{2+}]_i = K_d(F_0/F_s)(R - R_{min.})/(R_{max.} - R)$$

where $R_{min.}$ and $R_{max.}$ are the R values obtained without Ca^{2+} and with the saturating concentration of Ca^{2+}, respectively, K_d is the effective dissociation constant (224 nM), F_0 is the 380 nm excitation signal in the absence of Ca^{2+}, and F_s is the same signal with the saturating Ca^{2+} concentration.

Platelet protein phosphorylation

Platelets suspended in buffer containing 15 mM-Tris/HCl (pH 7.5), 0.14 M-NaCl and 1 mg of glucose/ml at a cell density of 3×10^9/ml were incubated with 0.5 mCi of carrier-free [^{32}P]P_i/ml for 60 min at 37 °C. The platelets were then diluted and washed twice. The [^{32}P]P_i-loaded platelets were further incubated with stimulants for 2 min, and the reaction was terminated by addition of Laemmli sample buffer [16], followed by boiling for 3 min. Proteins were then separated by SDS/PAGE (12.5% gels) [16]. After being dried, radioactive bands on the gel were detected by autoradiography. Where indicated, radioactivities in pleckstrin and the 20 kDa protein bands were quantified with a CS-9000 densitometer (Shimadzu Co., Kyoto, Japan).

PKC activity assay

PKC was partially purified from the cytosolic fraction of human platelets (5×10^{10} cells) with a DE-52 (Whatman) column as described previously [17]. PKC activity was assayed by measuring the incorporation of [^{32}P]P_i from [γ-^{32}P]ATP into histone H-1 (type III-S) by the method of Inagaki *et al.* [18]. The reaction mixture (0.2 ml) contained 25 mM-Tris/HCl (pH 7.0), 10 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, 50 μg of phosphatidylserine/ml, 10 mM-2-mercaptoethanol, 10 μM-[γ-^{32}P]ATP, 100 μg of histone III-S/ml and the enzyme preparation. The [^{32}P]P_i incorporation was corrected for by the background incorporation observed in the presence of 0.5 mM-EGTA instead of phosphatidylserine and $CaCl_2$ to obtain the phosphorylation selectively reflecting the PKC activity.

Translocation of PKC

Platelets (1×10^9 cells) suspended in 1 ml of Hepes–Tyrode's buffer were stimulated with 1 μM-PMA for 15 min at 37 °C. The platelets were then quickly spun down, washed once, and sonicated for 20 s in 1 ml of buffer A (20 mM-Tris/HCl, pH 7.4, 0.25 M-sucrose, 2 mM-EGTA, 50 mM-2-mercaptoethanol, 0.2 mM-phenylmethanesulphonyl fluoride, 100 μg of leupeptin/ml). The homogenates thus obtained were centrifuged at 100000 *g* for 60 min. The resulting supernatant served as the cytosolic fraction, and the pellet was dissolved in the same volume of buffer A containing 1% Triton X-100 and was used as the membrane fraction. These fractions were individually applied to 1 cm × 0.5 cm columns of DEAE-cellulose DE-52 equilibrated with buffer B (20 mM-Tris/HCl, pH 7.4, 2 mM-EGTA, 50 mM-2-mercaptoethanol, 10 μg of leupeptin/ml). After washing the columns with 15 vol. of buffer B, PKC was eluted batch-wise with 1 ml portions of 0.15 M-NaCl/buffer B. The eluates (20 μl) were assayed for PKC activity as described above.

[^{3}H]PDBu binding to platelets

[^{3}H]PDBu binding to whole platelets was performed as described by Hannun *et al.* [19].

RESULTS

Inhibition by wortmannin of platelet responses to various stimuli

Wortmannin (1 μM) inhibited the 5-hydroxytryptamine release induced by thrombin at lower concentrations, such as 0.02 unit/ml (Fig. 1*a*). The inhibition by wortmannin under these conditions was dependent on the concentration of this inhibitor used (Fig. 2*a*). The inhibition induced by wortmannin became smaller as the concentration of thrombin was increased above 0.02 unit/ml, until there was essentially no inhibition of 0.2 unit/ml (Fig. 1*a*). Similar results were obtained with platelets pretreated with aspirin, which only slightly inhibited thrombin-induced 5-hydroxytryptamine release (results not shown).

Collagen-induced release was markedly inhibited by 1 μM-wortmannin, especially when collagen concentration was maintained at 0.5 or 1.0 μg/ml (Fig. 1*b*). The inhibition observed with 1.0 μg of collagen/ml was dependent on the concentration of inhibitor added (Fig. 2*a*). The degree of wortmannin-induced inhibition became rather smaller as the concentration of collagen was increased to 2 and 5 μg/ml. In good agreement with the

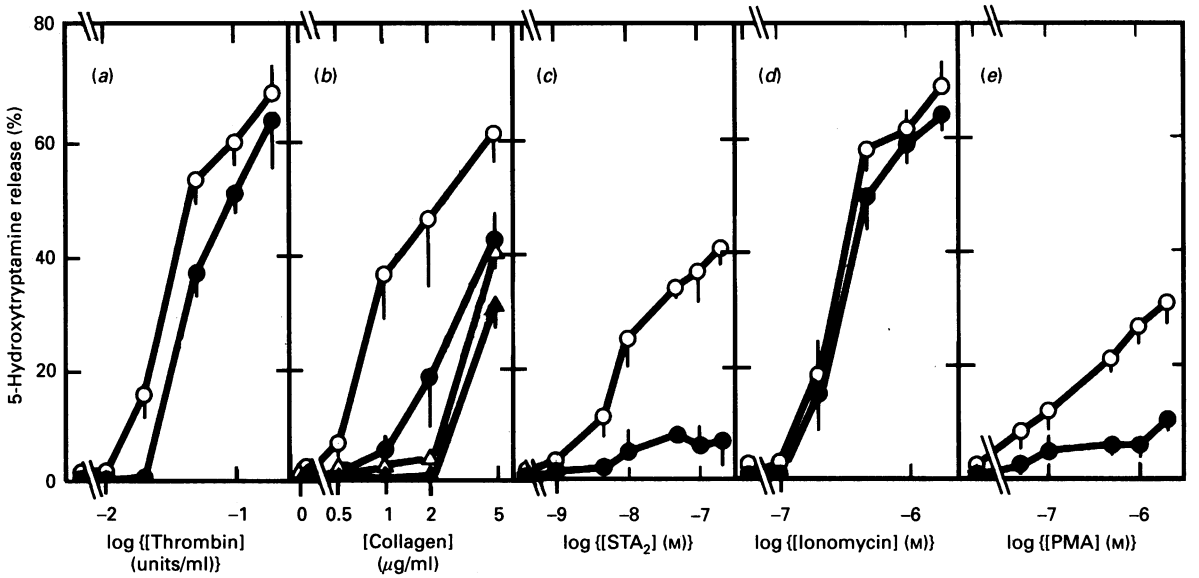

Fig. 1. Effects of wortmannin on the induction of 5-hydroxytryptamine release from platelets

After preincubation with 1 μM-wortmannin (black symbols) or vehicle (white symbols), platelet suspensions were stimulated with increasing concentrations of thrombin (*a*), collagen (*b*), STA_2 (*c*), ionomycin (*d*) or PMA plus 100 nM-ionomycin (*e*). The incubation time with stimulants was 5 min, except in (*e*), in which the incubation time was prolonged to 15 min. For collagen-induced 5-hydroxytryptamine release (*b*), the release from aspirin-treated platelets (triangles) is also plotted. Each point represents the mean ± S.E.M. for 3 or more observations.

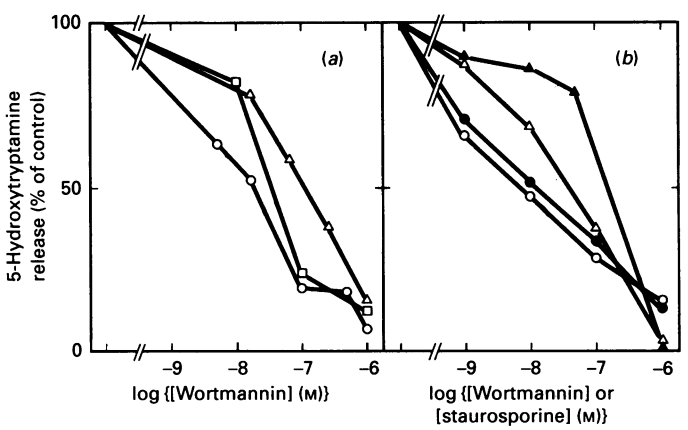

Fig. 2. Dose-dependent inhibition by wortmannin of 5-hydroxytryptamine release from platelets

In (*a*), the effects of various concentrations of wortmannin on 5-hydroxytryptamine release induced by 0.02 unit of thrombin/ml (○) (15.5%), 1 μg of collagen/ml (△) (36.1%) or 100 nM-STA_2 (□) (29.0%) are shown. In (*b*), the effects of various concentrations of wortmannin on 5-hydroxytryptamine release induced by 1 μM-PMA (○) (14.1%) or 1 μM-PMA plus 100 nM-ionomycin (●) (25.1%) are shown along with the effect of staurosporine on 5-hydroxytryptamine secretion induced by 1 μM-PMA (△) (16.7%) or 1 μM-PMA plus 100 nM-ionomycin (▲) (28.9%). The incubation times with stimulants were 5 min in (*a*) and 15 min in (*b*) respectively. Releases of 5-hydroxytryptamine are expressed as percentages of the values observed without the inhibitor, which are indicated in parentheses above. Results of a representative experiment are shown. At least two more experiments were performed for each stimulant, with essentially the same results.

notion that the effect of collagen on platelets is secondary to the production of thromboxane A_2 initiated by phospholipase A_2 activation [20,21], the 5-hydroxytryptamine release elicited by lower concentrations (below 2 μg/ml) of collagen was mostly abolished by pretreatment of the cells with aspirin. But further increase in the collagen concentration up to 5 μg/ml resulted in marked 5-hydroxytryptamine release, even in the presence of this cyclo-oxygenase inhibitor, which was only slightly inhibited by wortmannin.

5-Hydroxytryptamine release from platelets induced by STA_2, a stable analogue of thromboxane A_2 [22–24], was markedly prevented by 1 μM-wortmannin at any concentration of the agonist employed in either the absence (Fig. 1*c*) or the presence (results not shown) of aspirin, which had no effect on the STA_2-induced release reaction. The inhibition by wortmannin was dependent on its concentration (Fig. 2*a*).

Increase in the concentration of ionomycin above 200 nM gave rise to the concentration-dependent release of 5-hydroxytryptamine (Fig. 1*d*). The ionomycin-induced 5-hydroxytryptamine release was never inhibited by 1 μM-wortmannin at any concentration of the ionophore in either the absence (Fig. 1*d*) or the presence (results not shown) of aspirin, which partly inhibited the release induced by lower concentrations of ionomycin.

Only a small fraction of 5-hydroxytryptamine in the intracellular vesicles was released from platelets by addition of PMA alone; incubation of the platelet suspension with 1 μM-PMA for 5 min resulted in as little as 4.9 ± 0.6% 5-hydroxytryptamine release (mean ± S.E.M., $n = 3$). This small release was markedly inhibited by wortmannin in a dose-dependent manner even when the incubation time was prolonged to 15 min (Fig. 2*b*). PMA-induced 5-hydroxytryptamine release from human platelets was enhanced by the co-addition of a low concentration (100 nM) of ionomycin that did not induce the release by itself. This 5-hydroxytryptamine release by PMA plus ionomycin was also markedly inhibited by wortmannin (Fig. 1*e*) in a dose-dependent manner (Fig. 2*b*). Wortmannin was as potent as staurosporine (Fig. 2*b*), a powerful, although non-specific, PKC inhibitor [25], in inhibiting the PMA-induced 5-hydroxytryptamine release. Unexpectedly, H-7 [26] at concentrations higher than 100 μM or sphingosine [19,27] at concentrations higher than 50 μM alone induced 5-hydroxytryptamine release (results not shown), making it difficult to examine the effect of these PKC inhibitors on PMA-induced platelet 5-hydroxytryptamine release.

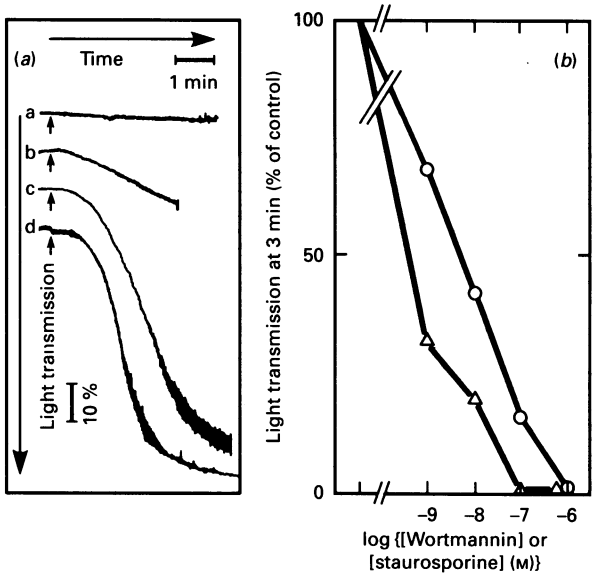

Fig. 3. PMA-induced platelet aggregation and its inhibition by wortmannin or staurosporine

In (*a*), representative aggregation traces by 1 nM- (a), 10 nM- (b), 100 nM- (c) and 1 μM- (d) PMA are shown. In (*b*), dose-dependent inhibition by wortmannin (○) or staurosporine (△) of 100 nM-PMA-induced aggregation is shown. Platelet aggregation is expressed as a percentage of light transmission at 3 min after addition of PMA without the inhibitor, which is 69.2±11.7% (mean±S.E.M., $n = 4$). Results of a representative experiment are shown. Similar results were obtained in at least two more experiments for each inhibitor.

The effect of wortmannin on PMA-induced aggregation of platelets was also studied. PMA at concentrations above 100 nM induced marked platelet aggregation even in the absence of ionomycin (Fig. 3*a*). The 100 nM-PMA-induced aggregation was inhibited dose-dependently by wortmannin as well as by staurosporine (Fig. 3*b*).

Failure by wortmannin to affect agonist-induced increases in $[Ca^{2+}]_i$

Thrombin [28,29] and STA_2 [23,24] are agonists of the receptors coupled to phospholipase C activation via G-proteins in platelets. Ca^{2+} mobilization induced by these agonists or a Ca^{2+} ionophore, ionomycin, was not inhibited by 1 μM-wortmannin at all (Fig. 4). Dibutyryl cyclic AMP (5 mM), used for comparison, inhibited $[Ca^{2+}]_i$ increases induced by receptor-mediated stimuli like those observed with thrombin and STA_2 (Fig. 4).

Effect of wortmannin on protein phosphorylation

It is established that when $[^{32}P]P_i$-labelled platelets are stimulated with PMA, pleckstrin is phosphorylated through PKC activation [5,10]. Furthermore, PMA is also known to phosphorylate a 20 kDa protein, which is the 20 kDa light chain of myosin [30], resulting from the action of PKC [30,31], rather than Ca^{2+}–calmodulin-dependent myosin light-chain kinase that is induced by thrombin or STA_2 [5,32,33]. Whereas pleckstrin was phosphorylated in PMA-stimulated platelets in a manner dependent on the concentrations of the phorbol ester from 1 nM up to 1 μM (Fig. 5*a*), phosphorylation of the 20 kDa protein was observed only when higher concentrations (above 100 nM) of PMA were employed. If a value of 100% was assigned to the degree of pleckstrin phosphorylation in the presence of 1 μM-PMA, the phosphorylation of the 20-kDa protein in the presence of 1 nM-, 10 nM-, 100 nM- and 1 μM-PMA was calculated to be 0.1±0.1%, 1.8±0.6%, 6.8±1.0% and 14.1±1.5% respectively (means±S.E.M., $n = 3$). The pleckstrin phosphorylation induced by 1 nM-PMA was inhibited by wortmannin in a concentration-dependent manner (Fig. 5*b*). The degree of wortmannin-induced inhibition became rather smaller as the concentration of PMA was increased above 10 nM, until there was no inhibition at 100 nM (Fig. 5*a*). Wortmannin failed to inhibit phosphorylation of the 20 kDa protein (Fig. 5*a*). Staurosporine [25,34] inhibited

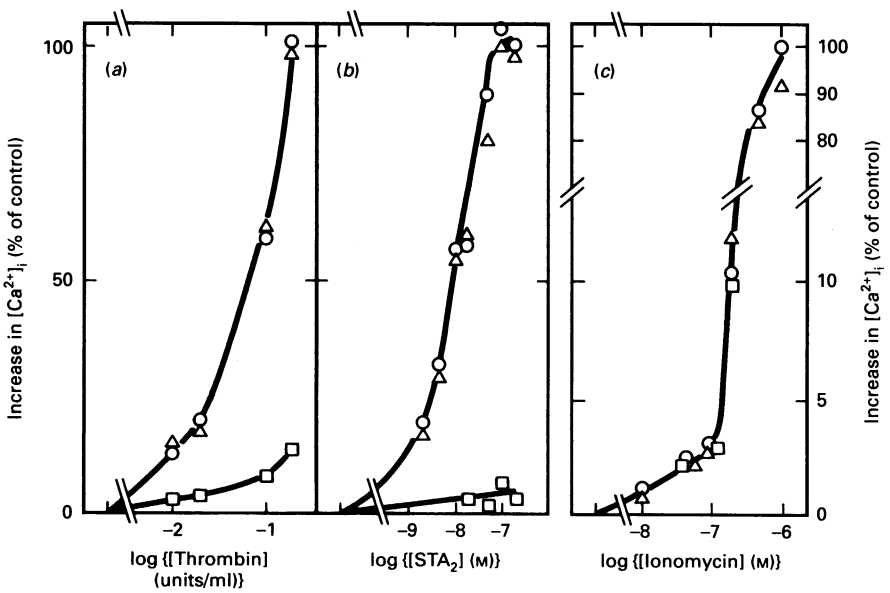

Fig. 4. Failure of wortmannin to inhibit increases in $[Ca^{2+}]_i$ triggered by thrombin (*a*), STA_2 (*b*) or ionomycin (*c*)

Fura2-loaded platelet suspensions pretreated with vehicle (○), 1 μM-wortmannin (△) or 5 mM-dibutyryl cyclic AMP (□) for 5 min were stimulated with various concentrations of stimulants. Increases in $[Ca^{2+}]_i$ are shown as percentages of the values observed without inhibitor after stimulation with 0.2 unit of thrombin/ml (*a*), 200 nM-STA_2 (*b*) or 1 μM-ionomycin (*c*), that was 1442–1538 nM, 327–666 nM or 5624–6099 nM respectively. Each line is a representative of three determinations.

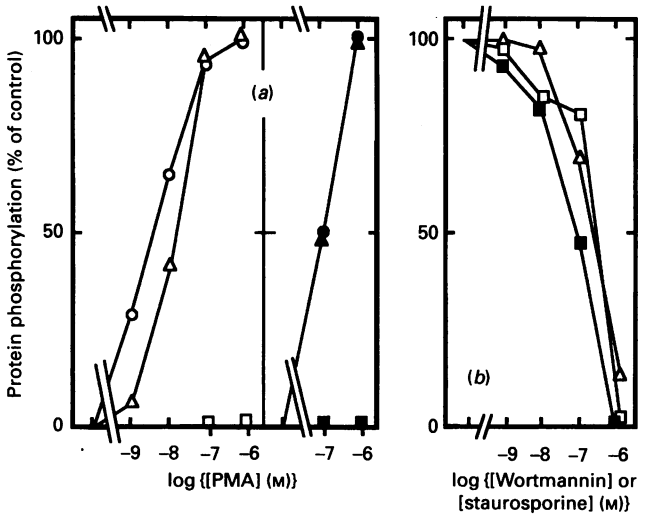

Fig. 5. Effects of wortmannin or staurosporine on phosphorylation of pleckstrin (white symbols) and a 20 kDa protein (black symbols) induced by PMA

In (*a*), [^{32}P]P_i-labelled platelets pretreated without inhibitor (○, ●) or with 1 μM-wortmannin (△, ▲) or 1 μM-staurosporine (□, ■) were stimulated with various concentrations of PMA. Platelet protein phosphorylation was evaluated by densitometry as described in the Materials and methods section. The optical densities with addition of 1 μM-PMA and without any addition were taken as 100% and 0% respectively. Each plot is the mean of 2 or 3 experiments. In (*b*), dose-dependent inhibition by wortmannin or staurosporine of platelet protein phosphorylation is shown. Platelet suspensions pretreated with various concentrations of wortmannin (△) or staurosporine (□, ■) were stimulated with 1 nM- or 1 μM-PMA respectively. The radioactivities are shown as percentages of the values observed after stimulation without the inhibitor. Each plot is the mean of 2 or 3 experiments.

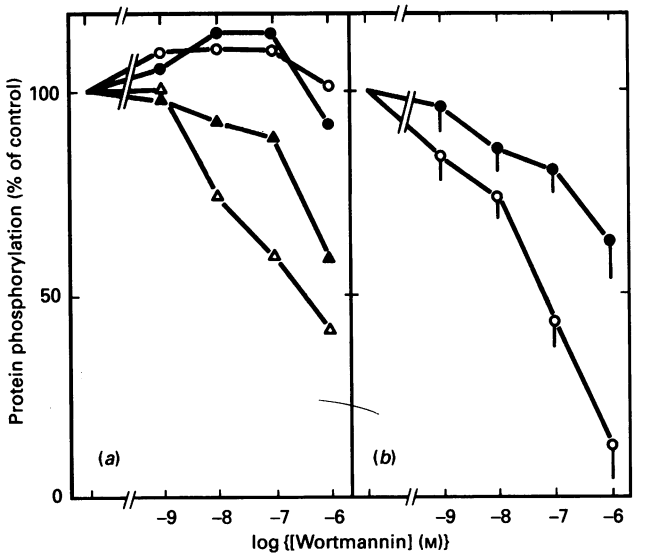

Fig. 6. Effects of wortmannin on platelet protein phosphorylation after stimulation with thrombin (*a*) or STA_2 (*b*)

Platelets preincubated with various concentrations of wortmannin were stimulated with 0.2 (○, ●) or 0.02 (△, ▲) unit of thrombin/ml in (*a*) or 100 nM-STA_2 (○, ●) in (*b*). The radioactivities are shown as percentages of the values observed without wortmannin. White and black symbols show radioactivity contents of pleckstrin and a 20 kDa protein respectively. Each point is the mean from 2 experiments or the mean ± S.E.M. from 3 determinations.

phosphorylation of both pleckstrin and the 20 kDa protein (Fig. 5*a*) concentration-dependently (Fig. 5*b*).

Thrombin and STA_2 phosphorylate both pleckstrin and the 20 kDa protein through the mediation of PKC and myosin light-chain kinase respectively [5,6]. Wortmannin inhibited the phosphorylation induced by 0.02 unit of thrombin/ml (Fig. 6*a*) or 100 nM-STA_2 (Fig. 6*b*), but did not inhibit the phosphorylation caused by 0.2 unit of thrombin/ml (Fig. 6*a*). These inhibitions were well correlated with the inhibitions of 5-hydroxytryptamine release induced by these receptor-mediated agonists (Figs. 1*a* and 1*c*). The agonist-induced phosphorylation of pleckstrin was inhibited by wortmannin more markedly than that of the 20 kDa protein (Fig. 6).

Lack of evidence for direct inhibition of PKC activity by wortmannin

The activity of PKC partially purified from the cytosolic fraction of human platelets, as measured by phosphorylation of histone by [γ-^{32}P]ATP, was inhibited by addition of staurosporine, but not at all by wortmannin under the conditions employed (Fig. 7).

The binding of PKC to membranes and activation of the kinase are considered to be separable events [35,36]. Effects of wortmannin on PKC translocation in platelets were therefore studied to gain an insight into the inhibition of pleckstrin phosphorylation. The PKC activity showed an activity change in the membrane and cytosolic fractions in a 'see-saw' fashion after incubation of platelets with PMA, presumably reflecting translocation of cytosolic PKC to membranes during the activation in intact cells (Figs. 8*a* and 8*b*). The translocation was not prevented

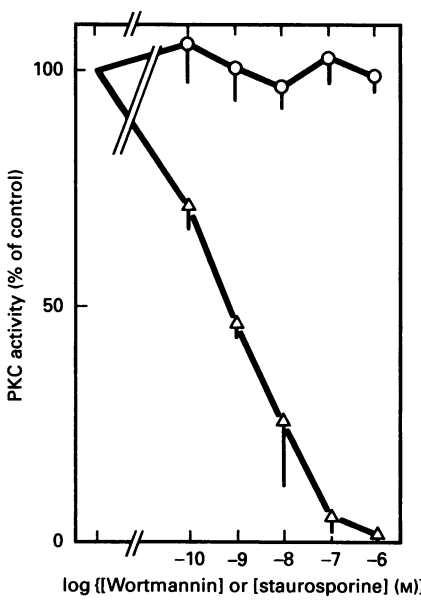

Fig. 7. Effects of wortmannin or staurosporine on the activity of PKC partially purified from platelet cytosolic fraction

PKC activity was assayed as described in the Materials and methods section in the presence of various concentrations of wortmannin (○) or staurosporine (△). The enzyme activity is expressed as a percentage of the control value obtained without inhibitor, 2598 ± 152 pmol of P_i incorporated/min per mg of protein (mean ± S.E.M., $n = 13$). Each point represents the mean ± S.E.M. from 3 or more determinations.

by the pretreatment of platelets with wortmannin (Fig. 8). Similarly negative results were obtained with its effect on [^{3}H]PDBu binding to platelets. Control [^{3}H]PDBu binding to platelets (100%) was not influenced at all by pretreatment of the

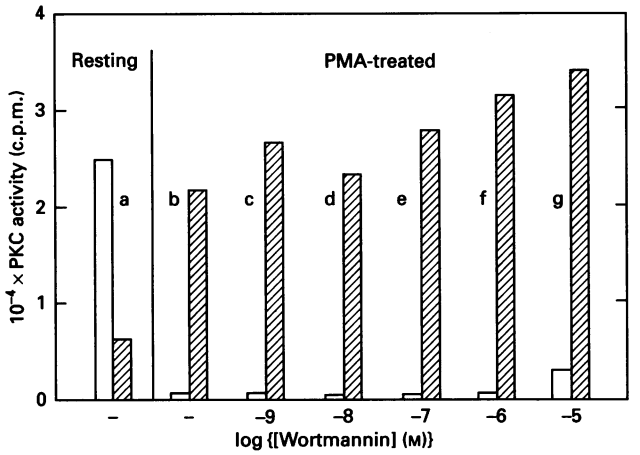

Fig. 8. Effect of wortmannin on translocation of PKC during incubation with PMA

Platelet suspensions preincubated without (a, b) or with various concentrations of wortmannin (c–g) were stimulated with 1 μM-PMA for 15 min (b–g). After the incubation, PKC activities of cytosolic (open column) and particulate (shaded columns) fractions were measured as described in the Materials and methods section. A representative result of 4 separate experiments is shown.

cells with 1 μM-wortmannin (110±7%; mean±S.E.M., $n = 3$) in spite of the inhibition caused by 100 μM-sphingosine (25±8%; mean±S.E.M., $n = 3$).

DISCUSSION

The potent inhibitory effects of wortmannin on platelet functions *in vitro* seem to be consistent with the previous finding that this fungal metabolite gave rise to severe haemorrhage in animals [2,3], although other mechanisms could be involved in the effect *in vivo*. Wortmannin inhibited 5-hydroxytryptamine release induced by thrombin, STA_2 or PMA, but did not inhibit the release by ionomycin (Fig. 1). The potency of wortmannin to inhibit PMA-induced 5-hydroxytryptamine release or aggregation could be comparable with that of staurosporine (Figs. 2*b* and 3*b*), a potent, although non-specific, PKC inhibitor [25]. These results suggest that its inhibitory effect results from its interaction with a PKC pathway; thrombin and STA_2 can activate PKC after receptor-mediated stimulation of phosphoinositide turnover [5]. Wortmannin did not inhibit $[Ca^{2+}]_i$ transients induced by any stimulant (Fig. 4), indicating that the site of its interaction is distal to the production of inositol trisphosphate or Ca^{2+} influx through plasma membranes. The inhibition by wortmannin of thrombin-induced 5-hydroxytryptamine discharge was reversed by increasing the concentration of thrombin up to 0.2 unit/ml (Fig. 1*a*). Conceivably, platelet activation induced by this agonist might be complicated by the existence of a pathway that is independent of phosphoinositide hydrolysis [29,37] and hence insensitive to wortmannin. The inhibitory effect of wortmannin on collagen-induced platelet secretion can be explained by its inhibition of the action of thromboxane A_2 which is produced after collagen stimulation of platelets [20,21]. In fact, wortmannin inhibited the 5-hydroxytryptamine release induced by STA_2, an analogue of thromboxane A_2 [22] (Fig. 1*c*).

We therefore propose that phosphorylation of pleckstrin, the well-known PKC substrate in platelets [5,8–11], plays an important role in triggering the cellular responses (e.g. 5-hydroxytryptamine release and aggregation) to various stimuli and that inhibition of the phosphorylation by wortmannin is a mechanism involved in inhibition of the cellular responses. There was a good correlation in the susceptibility to wortmannin-induced inhibition between the two parameters, i.e. the pleckstrin phosphorylation on the one hand and the cellular responses on the other. For instance, both parameters were inhibited by wortmannin, if both were initiated by 0.02 unit of thrombin/ml, but were no longer inhibited as the concentration of thrombin was increased up to 0.2 unit/ml (Figs. 1*a* and 6*a*). Both were also inhibited by wortmannin if STA_2 was used as a receptor agonist (Figs. 1*c* and 6*b*). These receptor agonists also caused phosphorylation of a 20 kDa protein, which is catalysed by Ca^{2+}–calmodulin-dependent myosin light-chain kinase [32,33]. The phosphorylation of the 20 kDa protein was less sensitive to wortmannin-induced inhibition than was phosphorylation of pleckstrin (Fig. 6).

PMA-induced phosphorylation of pleckstrin was also inhibited by wortmannin (Fig. 5). The disparity was noted, however, in the case of inhibitions of PMA actions, as follows. Inhibition by wortmannin of the protein phosphorylation became smaller as the concentration of PMA was increased up to 100 nM, where no inhibition was observed at all (Fig. 5*a*), despite the facts that PMA (plus ionomycin)-induced 5-hydroxytryptamine release was inhibited by wortmannin at all concentrations of PMA used (Fig. 1*e*) and that platelet aggregation induced by 100 nM-PMA was totally inhibited if the concentration of wortmannin was increased (Fig. 3*b*). A previous study with two-dimensional gel electrophoresis revealed the presence of two or three major forms of pleckstrin [9]. Another study revealed that the pattern of the PKC-catalysed platelet protein phosphorylation induced by PMA was different from that by receptor agonists [30]. It would therefore be likely that wortmannin inhibits the phosphorylation of only limited forms of pleckstrin which is selectively phosphorylated by PKC activated by receptor agonists and that PMA-activated PKC may phosphorylate other forms of pleckstrin which could be wortmannin-insensitive and unrelated to platelet functions. This postulate is compatible with the well-known fact that PMA-induced phosphorylation of pleckstrin is very strong, despite its weak effect on 5-hydroxytryptamine release [6].

PKC, which catalyses pleckstrin phosphorylation, could be considered to be the most probable candidate for the site where wortmannin interacts with the intracellular signalling pathway. Nevertheless, this fungal metabolite did not directly inhibit PKC activity in a cell-free conventional assay system [18] using partially purified PKC from platelet cytosol and histone as substrate, although the same assay system was highly sensitive to staurosporine (Fig. 7). Wortmannin added to an intact-cell preparation of platelets was also ineffective on the PMA-induced cytosolic PKC translocation to the membrane that may reflect an activation state of the protein kinase (Fig. 8). Conceivably, the fungal metabolite may interact with intact platelets, thereby inhibiting the PKC-induced phosphorylation of pleckstrin rather indirectly in such a manner that is not reflected in altered translocation of the cytosolic protein kinase. It would be possible to assume that wortmannin may produce a condition unfavourable for pleckstrin phosphorylation by PKC as a result of its interaction with the substrate protein in the intact cells.

Since the 47 kDa protein pleckstrin was discovered in platelets [8], the function of this PKC substrate has been controversial. Suggestions have been made for identification of this protein as lipocortin [38], inositol 1,4,5-trisphosphate 5-phosphomonoesterase [39], pyruvate dehydrogenase α subunit [40] or an actin regulatory protein [41]. Recently, cDNA for this protein was cloned from HL-60 cells [10]; the protein was found to be expressed in platelets and variously differentiated leucocytes selectively, but never in non-haematopoietic cells [10,11]. It differed distinctly from lipocortin or pyruvate dehydrogenase α subunit [10], and is not only expressed but also phosphorylated upon activation of PKC in leucocytes as well as in platelets [11].

The present results obtained with human platelets are at variance, at least in part, with those reported by Beatrice *et al.* [4] concerning inhibition by a derivative of wortmannin of the respiratory burst in neutrophils, in which pleckstrin is also expressed. They reported that the fungal metabolite inhibited receptor-mediated, but not PMA-induced, superoxide anion production. The inhibition was not associated with any change in $[Ca^{2+}]_i$, suggesting similar site(s) where wortmannin interacts with the cellular signalling pathway in neutrophils and platelets. The failure of wortmannin to inhibit PMA actions in neutrophils could be related to the fact that pleckstrin is the predominant substrate of PMA-stimulated PKC in platelets, but not so in neutrophils [11]. Further study may clarify the common mechanism underlying the different effects of wortmannin observed between these haematological cells.

This work was supported by a grant-in-aid for specially promoted research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Abbas, H. K. & Mirocha, C. J. (1988) Appl. Environ. Microbiol. **54**, 1268–1274
2. Abbas, H. K., Mirocha, C. J. & Gunther, R. (1989) Mycopathologia **105**, 143–151
3. Gunther, R., Abbas, H. K. & Mirocha, C. J. (1989) Food Chem. Toxicol. **27**, 173–179
4. Beatrice, D., Thelen, M. & Baggiolini, M. (1988) J. Biol. Chem. **263**, 16179–16184
5. Nishizuka, Y. (1984) Nature (London) **308**, 693–698
6. Siess, W. (1989) Physiol. Rev. **69**, 58–178
7. Rink, T. J. & Hallam, T. J. (1984) Trends Biochem. Sci. **9**, 215–219
8. Lyons, R. M., Stanford, N. & Majerus, P. W. (1975) J. Clin. Invest. **56**, 924–936
9. Imaoka, T., Lynham, J. A. & Haslam, R. J. (1983) J. Biol. Chem. **258**, 11404–11414
10. Tyers, M., Rachubinski, R. A., Stewart, M. I., Varrichio, A. M., Shorr, R. G. L., Haslam, R. J. & Harley, C. B. (1988) Nature (London) **333**, 470–473
11. Gailani, D., Fisher, T. C., Mills, D. C. B. & Macfarlane, D. E. (1990) Br. J. Haematol. **74**, 192–202
12. Higashihara, M., Maeda, H., Shibata, Y., Kume, S. & Ohashi, T. (1985) Blood **65**, 382–391
13. Legrand, C., Dubernard, V. & Nurden, A. T. (1985) Biochim. Biophys. Acta **812**, 802–810
14. Balduini, C. L., Bertolino, G., Noris, P., Sinigaglia, F., Bisio, A. & Torti, M. (1988) Biochem. Biophys. Res. Commun. **156**, 823–829
15. Cobbold, P. H. & Rink, T. J. (1987) Biochem. J. **248**, 313–328
16. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
17. Kajikawa, N., Kikkawa, U., Itoh, K. & Nishizuka, Y. (1989) Methods Enzymol. **169**, 430–442
18. Inagaki, M., Watanabe, M. & Hidaka, H. (1985) J. Biol. Chem. **260**, 2922–2925
19. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr. & Bell, R. M. (1986) J. Biol. Chem. **261**, 12604–12609
20. Sano, K., Takai, Y., Yamanishi, J. & Nishizuka, Y. (1983) J. Biol. Chem. **258**, 2010–2013
21. Nakano, T., Hanasaki, K. & Arita, H. (1989) J. Biol. Chem. **264**, 5400–5406
22. Ushikubi, F., Nakajima, M., Hirata, M., Okuma, M., Fujiwara, M. & Narumiya, S. (1989) J. Biol. Chem. **264**, 16496–16501
23. Hatayama, K., Kambayashi, J., Kawasaki, T., Morimoto, K., Ohshiro, T. & Mori, T. (1986) Thromb. Res. **41**, 761–770
24. Kawahara, Y., Fukuzaki, H., Kaibuchi, K., Tsuda, T., Hoshijima, M. & Takai, Y. (1986) Thromb. Res. **41**, 811–818
25. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) Biochem. Biophys. Res. Commun. **135**, 397–402
26. Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) Biochemistry **23**, 5036–5041
27. Hannun, Y. A., Greenberg, C. S. & Bell, R. M. (1987) J. Biol. Chem. **262**, 13620–13626
28. Billah, M. M. & Lapetina, E. G. (1982) J. Biol. Chem. **257**, 12705–12708
29. Brass, L. F., Laposata, M., Banga, H. S. & Rittenhouse, S. E. (1986) J. Biol. Chem. **261**, 16838–16847
30. Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) Nature (London) **306**, 490–492
31. Inagaki, M., Kawamoto, S. & Hidaka, H. (1984) J. Biol. Chem. **259**, 14321–14323
32. Adelstein, R. S. (1983) J. Clin. Invest. **72**, 1863–1866
33. Hathaway, D. R. & Adelstein, R. S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 1653–1657
34. Nakadate, T., Jeng, A. Y. & Blumberg, P. M. (1988) Biochem. Pharmacol. **37**, 1541–1545
35. Bazzi, M. D. & Nelsestuen, G. L. (1987) Biochemistry **26**, 115–126
36. Bazzi, M. D. & Nelsestuen, G. L. (1988) Biochem. Biophys. Res. Commun. **152**, 336–343
37. Lapetina, E. G., Silio, J. & Ruggiero, M. (1985) J. Biol. Chem. **260**, 7078–7083
38. Touqui, L., Rothhut, B., Shaw, A. M., Fradin, A., Vargaftig, B. B. & Russo-Marie, F. (1986) Nature (London) **321**, 177–180
39. Connolly, T. M., Lawing, W. J., Jr. & Majerus, P. W. (1986) Cell **46**, 951–958
40. Chiang, T. M., Kang, E. S. & Kang, A. H. (1987) Arch. Biochem. Biophys. **252**, 15–23
41. Hashimoto, K., Hashimoto, K., Im, K., Tatsumi, N., Okuda, K. & Yukioka, M. (1987) Biochem. Int. **14**, 759–767

Received 27 August 1991/29 January 1992; accepted 13 February 1992