Daphnoretin, a new protein kinase C activator isolated from *Wikstroemia indica* C.A. Mey.

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Daphnoretin, a biologically active principle isolated from *Wikstroemia indica* C.A. Mey., caused platelet aggregation in washed rabbit platelets, platelet-rich plasma and whole blood. The aggregation of and ATP release from platelets induced by daphnoretin were similar to phorbol ester- and diacylglycerol-induced aggregation and release. The EC_{50} values of daphnoretin-, phorbol 12,13-dibutyrate (PDBu)- and 1-oleoyl-2-acetylglycerol (OAG)-induced platelet aggregation in washed rabbit platelets were 17.2 ± 2.8 μM, 20.6 ± 2.1 nM and 38.6 ± 1.7 μM respectively. Platelet aggregation induced by daphnoretin and PDBu was not inhibited by indomethacin, BN52021 or sodium nitroprusside. ADP-scavenging systems, apyrase and phosphocreatine/creatine kinase, showed weak inhibition of the aggregation, and EGTA, triflavin, verapamil and prostaglandin E_1 markedly inhibited the aggregation. Staurosporine, a potent protein kinase C inhibitor, suppressed daphnoretin-, PDBu- and OAG-induced aggregation and ATP release in a concentration-dependent manner. The IC_{50} values of staurosporine on daphnoretin (50 μM)-, PDBu (100 nM)- and OAG (50 μM)-induced aggregation were 37.7 ± 8.3, 52.2 ± 6.3 and 42.8 ± 8.9 nM respectively. Daphnoretin did not cause significant thromboxane B_2 formation in rabbit platelets. Neither daphnoretin nor PDBu caused [^{3}H]inositol monophosphate formation or an increase in intracellular Ca^{2+} concentration in *myo*-[^{3}H]inositol-labelled and Fura-2-loaded platelets. Platelet cytosolic protein kinase C was activated by daphnoretin and PDBu in a concentration-dependent manner with an EC_{50} of 12.4 ± 1.2 μM and 18.7 ± 1.4 nM respectively. Membrane-associated protein kinase C activity was increased by either daphnoretin or PDBu. [^{3}H]PDBu binding to washed rabbit platelets was inhibited by daphnoretin in a concentration-dependent manner with an IC_{50} value of 45.2 ± 5.2 μM. These results indicate that daphnoretin is a protein kinase C activator in rabbit platelets.

INTRODUCTION

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent protein serine- and/or threonine-specific kinase [1]. This enzyme is activated directly by diacylglycerol produced by signal-induced inositol phospholipid turnover [2] and tumour promoters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-dibutyrate (PDBu) [3,4]. When cells are stimulated, an apparent translocation of PKC from the soluble fraction to the membrane occurs in a Ca^{2+}-dependent manner [5]. PKC has also been demonstrated to be the intracellular receptor for the tumour-promoting phorbol esters which activate the enzyme by interaction at the *sn*-1,2-diacylglycerol site [6–8]. PKC plays a central role in a wide variety of cell types. In platelets, it is supposed to be involved in the cellular activation process, which is initiated by the phosphorylation of specific proteins and results in granule release and aggregation [9–11].

Wikstroemia indica C.A. Mey. is a plant belonging to the Thymelaceae. The root of this plant is used as a folk remedy for arthritis, tuberculosis, syphilis and pertussis. The bicoumarin, daphnoretin (7-hydroxy-6-methoxy-3,7′-dicoumaryl ether), had been isolated from *Wikstroemia indica* C.A. Mey. [12] as well as *Daphne mezereum* L. and *Daphne cannabina* Wall [13]. Recently, we found that it induced aggregation of washed rabbit platelets in a manner similar to 1,2-diacylglycerols and phorbol esters. In this paper, we investigated the biological activity governed by daphnoretin in rabbit platelets. We also investigated the mechanism(s) of this activity and compared it with that of PDBu and 1-oleoyl-2-acetylglycerol (OAG).

EXPERIMENTAL

Materials

Daphnoretin (Figure 1) was isolated from the plant, *Wikstroemia indica* C.A. Mey., as described previously [12]. Collagen (type I, bovine Achilles tendon), from Sigma Chemical Co., was homogenized in 25 mM acetic acid and stored (1 mg/ml) at −70 °C. Bovine thrombin obtained from Parke Davis Co. was dissolved in glycerol (50 %, v/v) for a stock solution of 100 NIH units/ml. Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) purchased from Sigma was dissolved in chloroform and diluted into 0.1 % BSA/saline solution immediately before use. BSA, arachidonic acid, ADP, indomethacin, EDTA (disodium salt), EGTA, sodium citrate, luciferase–luciferin, apyrase, phosphocreatine, creatine kinase, verapamil, prostaglandin E_1 (PGE_1), sodium nitroprusside, trichloroacetic acid, histone (type III-S), L-α-phosphatidyl-L-serine, Dowex 1 (100–200 mesh: X8, Cl^-) resin and *myo*-inositol were purchased from Sigma. PDBu, OAG and staurosporine were obtained from Biomol Research Laboratory. *myo*-[2-^{3}H]Inositol (10–20 Ci/mmol), [20-^{3}H(n)]PDBu (10–20 Ci/mmol), [γ-^{32}P]ATP (triethylammonium salt, 3000 or 3 Ci/mmol) and PKC enzyme assay kit was purchased from Amersham. Thromboxane B_2 enzyme immunoassay kit was purchased from Cayman

Abbreviations used: PDBu, phorbol 12,13-dibutyrate; OAG, 1-oleoyl-2-acetylglycerol; PAF, platelet-activating factor; PKC, protein kinase C; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PGE_1, prostaglandin E_1; PRP, platelet-rich plasma; DMSO, dimethyl sulphoxide.

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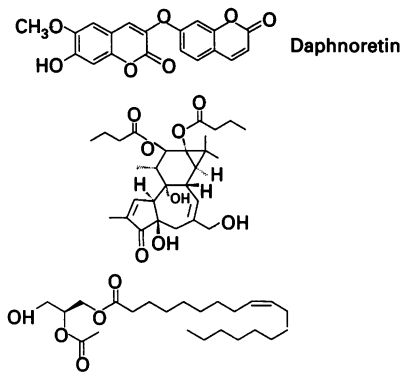

Figure 1 Chemical structures of daphnoretin, PDBu and OAG

Chemical Co. BN52021 (Ginkgolide B), a specific PAF antagonist isolated from the plant, *Ginkgo biloba* [14], was a gift from Dr. P. Braquet, Institute Henri Beaufour, France. Triflavin, a platelet fibrinogen receptor antagonist [15], was a gift from Dr. T. F. Huang, Pharmacological Institute, College of Medicine, National Taiwan University, Taiwan.

Preparation of platelets

Platelet-rich plasma (PRP) was obtained from blood collected from rabbit marginal vein, anticoagulated with sodium citrate (3.8%, 1:9, v/v) and centrifuged for 10 min at 90 *g* at room temperature. Platelet suspension was prepared from EDTA-anticoagulated PRP according to washing procedures described previously [16]. Platelets were counted by Hemalaser 2 (Sebia) and adjusted to a concentration of 3×10^8 platelets/ml. Platelet pellets were finally suspended in Tyrode's buffer (136.8 mM NaCl, 2.8 mM KCl, 11.9 mM $NaHCO_3$, 2.1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 1 mM $CaCl_2$, 11.2 mM glucose, pH 7.4) containing 0.35% BSA.

Platelet aggregation and ATP-release reaction

Aggregation was measured by the turbidimetry method as described by O'Brien [17]. ATP released from the platelets was detected by the bioluminescence method of DeLuca and McElory [18]. Both aggregation and ATP release were simultaneously measured in a Lumi-aggregometer (model 1020B; Payton) connected to two dual-channel recorders. Platelet preparations were stirred at 900 rev./min. When dimethyl sulphoxide (DMSO) was used as solvent, its final concentration was fixed at 0.5% (v/v) to eliminate the effect of the solvent.

Whole blood aggregometry

Blood anticoagulated with sodium citrate (3.8%, 1:9 v/v) was warmed up to 37 °C for 1 min in a whole blood aggregometer (model 500; Chrono-Log Co.); daphnoretin was then added to trigger the aggregation. The aggregation was reflected by the change in impedance between two platinum electrodes and recorded by a recorder.

Thromboxane B_2 assay

After 6 (for arachidonic acid, collagen, PAF and thrombin) or 20 (for daphnoretin) min of platelet activation with the inducer, 2 mM EDTA and 50 μM indomethacin were added to halt thromboxane formation. After centrifugation in an Eppendorf microcentrifuge (model 5415 C) for 2 min, thromboxane B_2 in the supernatant was assayed by enzyme immunoassay.

Measurement of $[Ca^{2+}]_i$ in platelets

The method of Pollock and Rink [19] was followed. Platelets (3×10^8/ml) were incubated with Fura-2/AM (5 μM) at 37 °C for 40 min, centrifuged at 500 *g* and the resultant pellet was washed with EDTA (1 mM)-containing Tyrode's solution. After centrifugation, platelets were resuspended in the Tyrode's solution containing 1 mM Ca^{2+}. Fluorescence (excitation wavelength 339 nm, emission wavelength 500 nm) was measured with a Hitachi fluorescence spectrophotometer (model F4000). At the end of the experiment the cells were treated with 0.1% Triton X-100 followed by the addition of 10 mM EGTA to obtain the maximal and minimal fluorescence respectively. $[Ca^{2+}]_i$ was calculated as described for Fura-2 using a Ca^{2+}–dye dissociation constant of 224 nM [20].

Labelling of membrane phospholipids and measurement of the production of [^{3}H]inositol phosphate

The method employed was modified from those of Huang and Detwiler [21] and Neylon and Summers [22]. EDTA/PRP was centrifuged at 500 *g* for 10 min. Platelet pellets were then suspended in 1 ml of Ca^{2+}-free and BSA-free Tyrode's solution containing *myo*-[2-^{3}H]inositol (75 μCi/ml) and EDTA (1 mM). After incubation for 2 h at 37 °C, the platelets were collected by centrifugation (1500 *g*, 4 min) and suspended in Tyrode's solution (3×10^8 platelets/ml). Phosphoinositide breakdown was enabled by addition of aggregation inducers to the platelet suspension (1 ml) in a 3.5 ml cuvette with a stirring bar driven at 1200 rev/min. Incubation was continued for either 6 or 20 min at 37 °C. An equal volume of 10% (w/v) trichloroacetic acid was then added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 ml of supernatant was aspirated and trichloroacetic acid removed by washing with diethyl ether. The aqueous phase containing inositol phosphates was adjusted to pH 7–8, diluted to 4 ml with distilled water, and applied to a Dowex 1 ion-exchange column for separation of inositol phosphates as described by Neylon and Summers [22]. [^{3}H]Inositol was eluted with 15 ml of 5 mM *myo*-inositol. [^{3}H]Glycerophosphoinositol was eluted with 30 ml of a buffer containing 5 mM sodium tetraborate and 60 mM sodium formate. [^{3}H]Inositol monophosphate, [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate were eluted with 20 ml of 0.2, 0.4 and 1.0 M ammonium formate/0.1 M formic acid. All experiments were carried out in the presence of 5 mM LiCl to inhibit inositol monophosphate phosphatase. Because concentrations of inositol bisphosphate and inositol trisphosphate were very low, we measured inositol monophosphate as an index of the total inositol phosphate formation.

Assay of PKC activity

The methods of Tapley and Murray [23] and Diagen et al. [24] were employed. Washed rabbit platelets prepared as described above were suspended at a final concentration of 10^9 cells/ml in Ca^{2+}-free Tyrode's solution. After incubation with DMSO, daphnoretin or PDBu at 37 °C for 20 min, platelets were collected by centrifugation (2000 *g*; 4 min; 4 °C), washed once with cold buffer and suspended (10^9 cells/ml) in a buffer (pH 7.5) containing sucrose (0.25 M), Hepes (20 mM), EDTA (2 mM), EGTA (5 mM), 2-mercaptoethanol (10 mM), leupetin (0.21 mM) and phenylmethanesulphonyl fluoride (2 mM), and disrupted by

sonication (4–6 × 10 s; setting 5, Vibra Cell, Sonics and Materials Inc.). The homogenate was centrifuged at 100000 *g* for 20 min (4 °C). The particulate fraction was resuspended in the same volume of above buffer containing 0.2 % Triton X-100, incubated at 4 °C for 1 h and centrifuged (100000 *g*; 30 min; 4 °C). After this, the supernatant was employed as a crude membrane extract. The crude extract was applied to a DEAE-cellulose DE-52 column (Whatman) in a volume of about 0.5 ml. The column was washed with 1.5 ml of equilibration buffer (20 mM-Tris/HCl, 0.5 mM-EGTA, 0.5 mM-EDTA, 10 mM-2-mercaptoethanol, pH 7.5) and 12.5 ml equilibration buffer containing 20 mM NaCl. Batchwise elution of PKC was achieved by applying 1.5 ml of equilibration buffer containing 120 mM NaCl to the column. After omission of the first 0.25 ml fraction, the remaining 1.25 ml was then taken as the fractions corresponding to PKC. PKC activity was measured using a commercially available kit (Amersham). The assay is dependent on PKC catalysing the transfer of radiolabelled ^{32}P from ATP to the threonine group of a peptide (a histone) which is specific for PKC. A linear incorporation of ^{32}P into the substrate peptide is observed.

In some experiments, homogenate from freshly prepared washed rabbit platelets was centrifuged at 100000 *g* for 20 min (4 °C), and the supernatant assayed directly for PKC activity as described by Daigen et al. [24]. Protein kinase assay mixture contained the enzyme preparation (30 μl), Hepes (20 mM, pH 7.5), [γ-^{32}P]ATP (3 nmol), $MgCl_2$ (5 mM), type III-S histone (20 μg), $CaCl_2$ (5 mM) and phosphatidylserine (20 μg/ml) in a final volume of 150 μl. Reactions were terminated by the addition of 1 ml of 25 % trichloroacetic acid. Trichloroacetate-precipitable materials were collected on a nitrocellulose membrane filter (Toyo-Roshi; pore size 0.45 μm) under vacuum. The filters were washed twice with 5 ml of 25 % trichloroacetic acid. Basal PKC activity was defined as the radioactivity in the absence of phosphatidylserine, Ca^{2+} and PKC activator (daphnoretin or PDBu). Also, 1 mM EGTA was substituted for Ca^{2+} in the assay tube for determination of basal activity.

PKC activity was expressed as pmol of ^{32}P incorporated into histone/min per 10^9 platelets.

[^{3}H]PDBu binding to rabbit platelets

Washed rabbit platelets were prepared as described above but resuspended at a concentration of 3×10^8 cells/ml in Ca^{2+}-free Tyrode's solution. Binding studies were performed by adding 100 nM [^{3}H]PDBu to a washed platelet suspension at room temperature; agitation was omitted in order to deter platelet aggregation. After 15 min of incubation with [^{3}H]PDBu, 400 μl portions were layered on the top of a sucrose solution (20 %, w/v) and centrifuged in an Eppendorf microcentrifuge [model 5415 C; 15000 *g* (14000 rev./min) for 5 min]. Tube bases containing sedimented platelet pellets were placed in vials containing Triton X-100 in 5 % HNO_3 (0.3 ml) for scintillation counting. Platelet pellets were then dislodged from the tube tips by vigorous agitation. Radioactivity was determined by liquid-scintillation counting after addition of a scintillation cocktail. Non-specific binding was defined in the presence of 50 μM unlabelled PDBu, and was substracted from total binding to afford specific binding.

RESULTS

Daphnoretin-, PDBu- and OAG-induced aggregation and release reaction

Daphnoretin, PDBu and OAG induced concentration-dependent aggregation of and ATP release from washed rabbit platelets (Figure 2). Compared with thrombin and collagen, daphnoretin, PDBu and OAG all caused a slow activation and aggregation of washed rabbit platelets (Table 1). Therefore percentage aggregation was calculated 20 min after these three aggregation inducers were added. The EC_{50} for daphnoretin-, PDBu- and OAG-induced platelet aggregation were 17.2 ± 2.8 μM, 20.6 ± 2.1 nM and 38.6 ± 1.7 μM with maximal effect at 50 μM, 100 nM and 50 μM respectively. These maximal effective concentrations were then used in the following experiments. Daphnoretin also caused aggregation of rabbit PRP and whole blood with EC_{50} 40.2 ± 1.2 and 17.3 ± 2.8 μM respectively.

Effect of some pharmacological agents on the platelet aggregation induced by daphnoretin, PDBu and OAG

In the presence of indomethacin (10 μM) and BN52021 (20 μM), arachidonic acid (100 μM)- and PAF (3.6 nM)-induced platelet aggregations were completely inhibited. However, indomethacin and BN52021 did not inhibit daphnoretin (50 μM)- and PDBu (100 nM)-induced aggregation in washed rabbit platelets (Table 2). Apyrase and phosphocreatine/creatine kinase were used as an ADP-scavenging system to study the effect of ADP released from platelets on the aggregation induced by daphnoretin and PDBu. Exogenous addition of ADP (20 μM) could not induce platelet aggregation in the presence of apyrase (1 unit/ml) or phosphocreatine/creatine kinase (5 mM/8 units per ml). Both daphnoretin- and PDBu-induced aggregations were partially suppressed by apyrase (1 unit/ml) and phosphocreatine/creatine kinase (5 mM/8 units per ml). When the concentrations of apyrase and phosphocreatine/creatine kinase were doubled, they did not cause more pronounced inhibition in daphnoretin- and PDBu-induced aggregation (Table 2). Among the pharmacological agents tested, EGTA (3 mM), verapamil (50 μM), triflavin (20 μg/ml) and PGE_1 (1 and 10 μM) all inhibited daphnoretin- and PDBu-induced aggregation markedly but incompletely (Table 2). Nitroprusside (10 μM) did not inhibit daphnoretin- and PBDu-induced platelet aggregation (Table 2). Staurosporine, a potent PKC inhibitor [25], inhibited daphnoretin-, PDBu- and OAG-induced aggregation completely in a concentration-dependent manner with IC_{50} 37.7 ± 8.3, 52.2 ± 6.3 and 42.8 ± 8.9 nM respectively (Figure 3).

Thromboxane B_2 formation in platelets treated with daphnoretin

The thromboxane B_2 level of resting platelets (3×10^8/ml) was 1.6 ± 0.4 ng/ml. Arachidonic acid (100 μM), collagen (10 μg/ml) and thrombin (0.1 unit/ml) markedly increased the thromboxane B_2 level after stimulation of platelets for 6 min. PAF (3.6 nM) also caused a slight increase in platelet thomboxane B_2 level. At the concentration used for inducing maximal aggregation of platelets, daphnoretin (50 μM) did not cause significant thromboxane B_2 formation when compared with the basal level at resting state (Table 3).

[^{3}H]Inositol monophosphate formation in platelets treated with daphnoretin and PDBu

The [^{3}H]inositol monophosphate levels of resting platelets incubated with LiCl (5 mM) for 6 and 20 min were 511 ± 40 and 633 ± 41 c.p.m./3×10^8 platelets respectively. After challenge with platelets for 6 min, thrombin (0.1 unit/ml), PAF (3.6 nM) and collagen (10 μg/ml) increased [^{3}H]inositol monophosphate formation 5.4 ± 0.7-, 3.1 ± 0.3- and 2.9 ± 0.2-fold respectively. Daphnoretin (50 μM) challenged with platelets for either 6 or 20 min did not cause any significant increase in platelet [^{3}H]-inositol monophosphate level (Figure 4). PDBu (100 nM) also

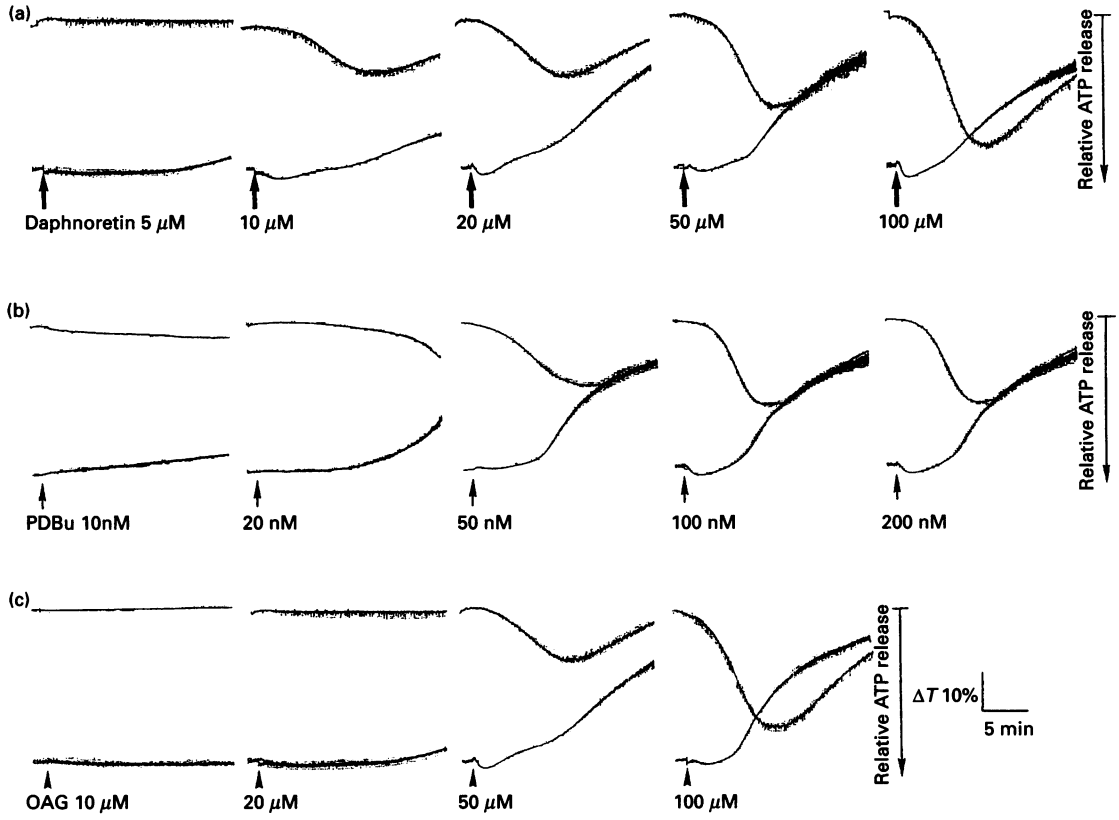

Figure 2 Aggregation of and ATP release from washed rabbit platelets induced by daphnoretin, PDBu and OAG

Platelets were warmed to 37 °C for 1 min in the presence of luciferase and luciferin, then daphnoretin (**a**), PDBu (**b**) or OAG (**c**) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

Table 1 Latent periods and times needed to achieve 50% aggregation of daphnoretin-, PDBu-, OAG-, thrombin- and collagen-induced platelet activation

Washed rabbit platelets were prewarmed to 37 °C for 1 min, then daphnoretin, PDBu, OAG, thrombin or collagen was added to trigger the aggregation. Values are expressed as means ± S.E.M. (*n*).

	Latent period (min)	50% Aggregation (min)
Daphnoretin (50 μM)	4.4 ± 0.4 (18)	9.5 ± 0.7 (18)
PDBu (100 nM)	4.7 ± 0.5 (12)	10.8 ± 0.9 (12)
OAG (50 μM)	3.9 ± 0.4 (5)	7.2 ± 0.7 (5)
Thrombin (0.1 unit/ml)	0.22 ± 0.02 (11)	0.35 ± 0.02 (11)
Collagen (10 μg/ml)	0.54 ± 0.04 (11)	0.84 ± 0.05 (11)

had no effect on platelet [^{3}H]inositol monophosphate formation (results not shown).

$[Ca^{2+}]_i$ level in platelets treated with daphnoretin and PDBu

In Fura-2-loaded platelets, the resting $[Ca^{2+}]_i$ was 63.8 ± 6.2 nM (n = 4). Thrombin (0.1 unit/ml) markedly increased the $[Ca^{2+}]_i$ of Fura-2-loaded platelets (393 ± 51.1 nM, n = 4). Daphnoretin (50 μM) and PDBu (100 nM) did not induce significant increase in $[Ca^{2+}]_i$ in the Fura-2-loaded platelets (results not shown).

Table 2 Effects of some pharmacological agents on the platelet aggregation induced by daphnoretin and PDBu

Washed rabbit platelets were preincubated with DMSO (0.5%; control) or various pharmacological agents at 37 °C for 3 min, then daphnoretin (50 μM) or PDBu (100 nM) was added to trigger the aggregation. Values are expressed as means ± S.E.M. (n = 5–7). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the respective control.

	Aggregation (%)	
	Daphnoretin	PDBu
Control	84.7 ± 1.1	82.2 ± 2.9
+ Indomethacin (10 μM)	83.9 ± 2.2	74.6 ± 3.6
+ BN52021 (20 μM)	85.0 ± 2.1	78.4 ± 2.9
+ Apyrase (1 unit/ml)	49.4 ± 9.0**	52.3 ± 4.1***
+ Apyrase (2 units/ml)	56.8 ± 5.9***	53.6 ± 4.9***
+ Phosphocreatine/creatine kinase (5 mM/8 units per ml)	45.2 ± 6.6***	47.1 ± 4.9***
+ Phosphocreatine/creatine kinase (10 mM/16 units per ml)	50.4 ± 6.5***	53.6 ± 4.9***
+ EGTA (3 mM)	16.4 ± 3.1***	24.8 ± 2.9***
+ Verapamil (50 μM)	34.5 ± 6.5***	23.3 ± 6.0***
+ Nitroprusside (10 μM)	79.6 ± 4.8	76.5 ± 2.4
+ Triflavin (20 μg/ml)	22.3 ± 3.1***	35.3 ± 1.2***
+ PGE_1 (1 μM)	23.9 + 8.2***	23.7 ± 3.1***
+ PGE_1 (10 μM)	28.4 ± 5.5***	19.0 ± 1.9***

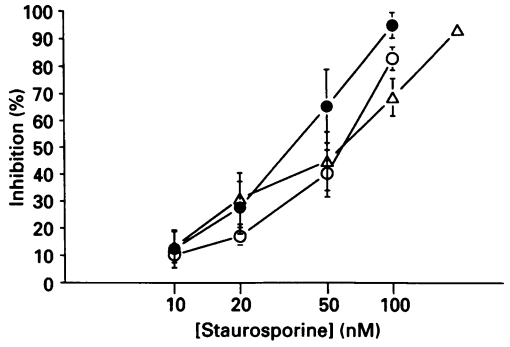

Figure 3 Inhibitory effect of staurosporine on platelet aggregation induced by daphnoretin, PDBu and OAG

Washed rabbit platelets were preincubated with DMSO (0.5%, control) or various concentrations of staurosporine at 37 °C for 3 min, then daphnoretin (50 μM, ●), PDBu (100 nM, ○) or OAG (50 μM, △) was added to trigger the aggregation. Percentages of inhibition are presented as means ± S.E.M. ($n = 5$).

Table 3 Thromboxane B_2 formation in washed rabbit platelets induced by daphnoretin, arachidonic acid, collagen, thrombin and PAF

The thromboxane B_2 level of resting platelets was 1.6 ± 0.4 ng/ml. Values are expressed as means ± S.E.M. ($n = 5$). *$P < 0.001$ compared with the resting level.

Aggregation inducer	[Thromboxane B_2] (ng/ml)
Daphnoretin (50 μM)	2.6 ± 0.5
Arachidonic acid (100 μM)	845 ± 52*
Collagen (10 μg/ml)	400 ± 12*
Thrombin (0.1 unit/ml)	164 ± 17*
PAF (3.6 nM)	10.0 ± 2.0*

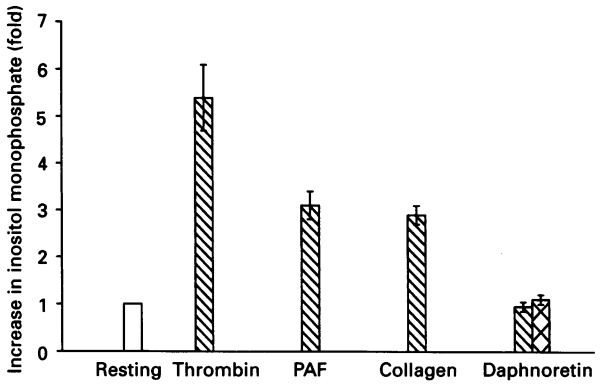

Figure 4 [^{3}H]Inositol monophosphate formation of washed rabbit platelets caused by thrombin, PAF, collagen and daphnoretin

[^{3}H]Inositol-labelled platelets were prewarmed to 37 °C for 1 min, then thrombin (0.1 unit/ml), PAF (3.6 nM), collagen (10 μg/ml) or daphnoretin (50 μM) was added for either 6 (▨) or 20 (⊠) min. Increases in inositol monophosphate are presented as means ± S.E.M. ($n = 4$).

Activation of platelet PKC by daphnoretin and PDBu

PKC from platelet cytosol was activated by daphnoretin (2–100 μM) and PDBu (5–100 nM) in a concentration-dependent manner. The maximal activities caused by daphnoretin and PDBu were 27.4 ± 2.1 and 29.7 ± 2.5 pmol/min per 10^9 platelets ($n = 4$) with EC_{50} 12.4 ± 1.2 μM and 18.7 ± 1.4 nM respectively. Staurosporine (100 nM) almost completely inhibited daphnoretin (100 μM)- and PDBu (100 nM)-induced PKC activity (1.2 ± 0.3 and 0.9 ± 0.2 pmol/min per 10^9 platelets respectively).

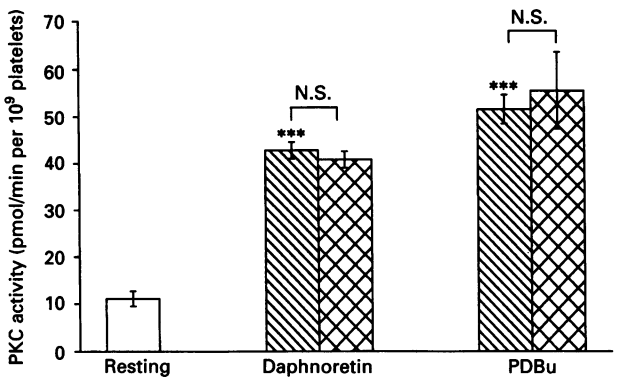

Figure 5 Membrane-associated PKC activity of washed rabbit platelets

Platelets were preincubated with 0.5% DMSO (□, resting; ▨, control) or staurosporine (100 nM; ⊠) at 37 °C for 3 min; then daphnoretin (50 μM) or PDBu (100 nM) was added for another 20 min. The membrane-bound PKC activity was determined as described in the Experimental section. Data are expressed as means ± S.E.M. ($n = 5$). ***$P < 0.001$ compared with the resting level. N.S., not significant.

Table 4 Effect of daphnoretin on specific [^{3}H]PDBu binding to intact rabbit platelets

Washed rabbit platelets were preincubated with DMSO (0.5%, control), PDBu (50 μM, for non-specific binding) or various concentrations of daphnoretin at room temperature for 1 min, then [^{3}H]PDBu (100 nM) was added for another 15 min. Non-specific [^{3}H]PDBu binding was 6995 ± 646 c.p.m., and was subtracted from total binding to afford specific binding. Specific [^{3}H]PDBu binding values are presented as means ± S.E.M. ($n = 6$).

	Specific [^{3}H]PDBu binding (c.p.m.)
Control	52330 ± 4387
Daphnoretin 10 μM	48770 ± 4514
20 μM	39889 ± 5288
50 μM	27298 ± 4001
100 μM	5857 ± 1172
200 μM	1073 ± 448

Translocation of PKC in platelets treated with daphnoretin and PDBu

The membrane-associate PKC activity of resting platelets was 11.1 ± 1.6 pmol/min per 10^9 platelets. Daphnoretin (50 μM) and PDBu (100 nM) markedly increased the membrane-associated PKC activity. Staurosporine (100 nM) almost completely inhibited daphnoretin- and PDBu-induced aggregation without having any effect on daphnoretin- and PDBu-induced PKC translocation from platelet cytosol to membrane (Figure 5).

Effect of daphnoretin on [^{3}H]PDBu binding

Total binding of 100 nM [^{3}H]PDBu to intact rabbit platelets in the absence of Ca^{2+} was about 59325 c.p.m., whereas non-

specific binding assayed in the presence of 50 μM PDBu was about 6995 c.p.m. Accordingly, under this study condition, about 88% of total binding of [^{3}H]PDBu was specific. Daphnoretin (10–200 μM) inhibited specific [^{3}H]PDBu binding to intact rabbit platelets in a concentration-dependent manner with IC_{50} 45.2 $\pm$ 5.2 μM (Table 4). Staurosporine (100 nM) had no effect on specific [^{3}H]PDBu binding to rabbit platelets (results not shown).

DISCUSSION

Platelet aggregation requires exposure of fibrinogen receptors to which fibrinogen binds in the presence of bivalent cations [26]. Chelation of extracellular Ca^{2+} inhibits the binding of fibrinogen to its receptor [27]. EGTA is a Ca^{2+} chelator which inhibits platelet aggregation by reducing extracellular free Ca^{2+} and thus prevents fibrinogen binding to its receptor. Triflavin, an RGD-containing peptide isolated from *Trimeresurus flavoviridis* snake venom, inhibits platelet aggregation by interfering with fibrinogen binding to its receptor associated with the glycoprotein IIb–IIIa complex [15]. Cyclic AMP is one of the most potent platelet inhibitors. Elevated cyclic AMP inhibits most platelet responses including aggregation. PGE_1 inhibits platelet aggregation by elevating the platelet cyclic AMP level [28]. In our experiments, Daphnoretin and PDBu increased the light transmittance of washed rabbit platelets. The increase in light transmittance was markedly blocked by EGTA, triflavin and PGE_1. Lactate dehydrogenase activity in the supernatant of platelet suspension challenged with daphnoretin was not increased (results not shown). All these data indicate that daphnoretin induced aggregation of rabbit platelets but not cell lysis or agglutination.

Like PDBu and OAG, but not other platelet stimuli, daphnoretin induced slow platelet responses (Figure 2 and Table 1). Daphnoretin- and PDBu-induced platelet aggregation also possessed similar susceptibility to some pharmacological agents. Thromboxane A_2, ADP and PAF are three important mediators usually formed and/or liberated from platelets by aggregation inducers such as thrombin and collagen, thereby amplifying platelet responses. Indomethacin, which inhibits the platelet cyclo-oxygenase and prevents the formation of thromboxane A_2, did not inhibit platelet aggregation caused by daphnoretin and PDBu. Daphnoretin also did not induce thromboxane B_2 formation in platelets. These data indicate that daphnoretin- and PDBu-induced platelet aggregation was independent of thromboxane formation. These results are also consistent with previous reports that diacylglycerols and phorbol esters do not induce arachidonate release and formation of arachidonate metabolites [29–32]. BN52021, a specific PAF antagonist, did not affect daphnoretin- and PDBu-induced platelet aggregation. This result implies that PAF also does not mediate daphnoretin- and PDBu-induced rabbit platelet aggregation. However, apyrase (1 unit/ml) and phosphocreatine/creatine kinase (5 mM/8 units per ml), two ADP-scavenging enzyme systems, slightly suppressed daphnoretin- and PDBu-induced platelet aggregation. Doubling the concentration of apyrase or phosphocreatine/creatine kinase did not cause additional inhibition. Thus released ADP plays only a minor role in mediating daphnoretin- and PDBu-induced aggregation of rabbit platelets. It has been reported that verapamil non-specifically inhibited PKC activity by competition with phospholipid [33]. Staurosporine is the most potent inhibitor which binds near the catalytic site of PKC [25]. Both daphnoretin- and PDBu-induced aggregations were susceptible to verapamil. Staurosporine also blocked daphnoretin-, PDBu- and OAG-induced platelet aggregation and ATP release in a concentration-dependent manner (Figure 3). These data strongly suggest that daphnoretin, like PDBu and OAG, may induce platelet responses by stimulating PKC activity.

In resting platelets, PKC is present in an inactive and soluble form in the cytosol. This enzyme is activated directly by diacylglycerol produced by signal-induced inositol phospholipid turnover [2] and phorbol esters such as TPA and PDBu [3,4]. After that, PKC shifts from the cytosol to the membrane [34]. As phosphoinositide breakdown is observed in platelets activated by many agonists, this process may be a primary event in agonist-induced activation [35,36]. Diacylglycerols and phorbol esters do not induce inositol phospholipid hydrolysis in platelets [29–32]. Diacylglycerols and phorbol esters also do not evoke a quin-2 [37], Fura-2 [38] or indo-1 [39] response in intact platelets. Daphnoretin and PDBu caused translocation of PKC from platelet cytosol to membrane. Daphnoretin and PDBu neither caused inositol phospholipid hydrolysis in *myo*-[^{3}H]inositol-labelled platelets (Figure 5) nor increased $[Ca^{2+}]_i$ in Fura-2-loaded platelets. These data imply that daphnoretin, like phorbol esters and diacylglycerols, did not activate phospholipase C in platelets. Thus daphnoretin might directly bind to PKC and activate the enzyme.

All the data mentioned above strongly suggest that daphnoretin directly activated platelet PKC. It is well known now that tumour-promoting phorbol esters, such as PDBu and TPA, have a diacylglycerol-like structure. They bind to PKC and activate it [3]. PKC and phorbol ester receptors seem to be identical [6,40]. One molecule of phorbol ester binds to one molecule of PKC. Specific binding of phorbol esters to intact platelets may therefore reflect the presence of PKC in the plasma membrane [39,41]. Specific [^{3}H]PDBu binding to intact rabbit platelets was high (about 88%). Staurosporine had no effect on specific [^{3}H]PDBu binding. This result was consistent with the previous report that staurosporine did not interfere with the binding of [^{3}H]PDBu [25]. Daphnoretin inhibited the specific [^{3}H]PDBu binding to intact rabbit platelets in a concentration-dependent manner (Table 4). In addition, PKC from the platelet cytosol was activated by either daphnoretin or PDBu in a concentration-dependent manner. These results suggest that daphnoretin and PDBu bind to the same site of PKC and activate the enzyme directly.

Our results strongly suggest that daphnoretin is a rabbit platelet PKC activator. Further investigation of its tumour-promoting activity is warranted.

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