Regulation of *sn*-1,2-diacylglycerol second-messenger formation in thrombin-stimulated human platelets

Potentialiation by protein kinase C inhibitors

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Stimulation of platelets with thrombin leads to rapid degradation of inositol phospholipids, generation of diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC). Previous studies indicated that prior activation of PKC with phorbol myristate acetate (PMA) desensitizes platelets to thrombin stimulation, as indicated by a decreased production of inositol phosphates and decreased Ca^{2+} mobilization. This suggests that PKC activation generates negative-feedback signals, which limit the phosphoinositide response. To test this hypothesis further, we examined the effects of PKC activators and inhibitors on thrombin-stimulated DAG mass formation in platelets. Pretreatment with PMA abolishes thrombin-stimulated DAG formation (50% inhibition at 60 nM). Pretreatment of platelets with the PKC inhibitors K252a or staurosporine potentiates DAG production in response to thrombin (3–4-fold) when using concentrations required to inhibit platelet PKC (1–10 μM). K252a does not inhibit phosphorylation of endogenous DAG or phosphorylation of a cell-permeant DAG in unstimulated platelets, indicating that DAG over-production is not due to inhibition of DAG kinase. Sphingosine, a PKC inhibitor with a different mechanism of action, also potentiates DAG formation in response to thrombin. Several lines of evidence indicate that DAG formation under the conditions employed occurs predominantly by phosphoinositide (and not phosphatidylcholine) hydrolysis: (1) PMA alone does not elicit DAG formation, but inhibits agonist-stimulated DAG formation; (2) thrombin-stimulated DAG formation is inhibited by neomycin (1–10 mM) but not by the phosphatidate phosphohydrolase inhibitor propranolol; and (3) no metabolism of radiolabelled phosphatidylcholine was observed upon stimulation by thrombin or PMA. These data provide strong support for a role of PKC in limiting the extent of platelet phosphoinositide hydrolysis.

INTRODUCTION

The primary event following binding of many extracellular agonists to their specific receptors is the hydrolysis of membrane phospholipids. The best-characterized pathway of phospholipid hydrolysis is the cleavage of the phosphoinositides phosphatidylinositol 4,5-bisphosphate (PtdInsP_2), phosphatidylinositol 4-phosphate and phosphatidylinositol (PtdIns) by a phospholipase C (PLC) mechanism (reviewed by Majerus *et al.*, 1986). PtdInsP_2 breakdown generates two second messengers: (i) inositol 1,4,5-trisphosphate (InsP_3), which mobilizes Ca^{2+} from intracellular stores; and (ii) *sn*-1,2-diacylglycerol (DAG), which remains membrane-associated and activates protein kinase C (PKC). Coupling of receptors to phosphoinositide-specific PLC appears to be mediated by a guanine-nucleotide-binding protein (G-protein).

There is considerable evidence that treatment of cells with phorbol esters inhibits subsequent phosphoinositide hydrolysis. For example, pretreatment with phorbol myristate acetate (PMA) blocks chemotactic-peptide- and guanosine 5'-[γ -thio]triphosphate-induced InsP_3 formation in plasma membranes from human polymorphonuclear leucocytes (Smith *et al.*, 1987) and in HL60 cells (Kikuchi *et al.*, 1987) and guanosine 5'-[γ -thio]triphosphate-plus-carbachol-induced InsP_3 formation in astrocytoma membranes (Orellana *et al.*, 1987). In all cases, PMA does not affect the ability of Ca^{2+} to activate PtdInsP_2 hydrolysis. Similarly, PMA blocks Ca^{2+} mobilization and InsP_3 production in hepatocytes stimulated with Ca^{2+} -mobilizing hormones or AlF_4^- (to activate the coupling G-protein directly)

(Blackmore & Exton, 1986). These observations led to the hypothesis that activation of PKC limits the phosphoinositide response by inhibiting the coupling of the G-protein to phosphoinositide-specific PLC.

Considerable work has been done on phosphoinositide turnover in thrombin-stimulated platelets. Hydrolysis of PtdInsP_2 is one of the earliest events on treatment of platelets with thrombin (Billah & Lapetina, 1982). PtdInsP_2 mass decreases transiently upon thrombin stimulation, reaching a minimum at 20 s and then returning to basal levels (Wilson *et al.*, 1985). A larger decrease in PtdIns mass is observed over the course of 90 s (Wilson *et al.*, 1985). Phosphoinositide hydrolysis is accompanied by accumulation of inositol phosphates. InsP_3 accumulation is transient; within 15 s InsP_3 levels reach 134 pmol/ 10^9 platelets, but then fall rapidly back towards basal levels by 2 min (Rittenhouse & Sasson, 1985). The other product of the PLC reaction, DAG, also accumulates transiently. DAG mass reaches a peak at 30 s (at 250–300 pmol/ 10^9 platelets), followed by a rapid decrease to basal levels (75–100 pmol/ 10^9 platelets) (Preiss *et al.*, 1986; Bishop *et al.*, 1986). The pathways for rapid attenuation of these second messengers have been investigated extensively (reviewed by Majerus *et al.*, 1986; Bishop & Bell, 1988).

PMA pretreatment also prevents phosphoinositide hydrolysis in platelets. PMA blocks thrombin-induced InsP_3 formation (Rittenhouse & Sasson, 1985; Watson & Lapetina, 1985) and Ca^{2+} mobilization (Zavoico *et al.*, 1985), consistent with a negative-feedback role for PKC in this system.

The objective of the present work was to test further the hypothesis that PKC activation limits the phosphoinositide

Abbreviations used: DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PC, phosphatidylcholine; PA, phosphatidic acid; PLC, phospholipase C; diC_8 , *sn*-1,2-dioctanoylglycerol; DMSO, dimethyl sulphoxide; IC_{50} , concn. giving 50% inhibition.

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response in platelets, by examining the effects of PKC activators and inhibitors on DAG formation. We found that phorbol ester pretreatment blocks DAG formation. In addition, PKC inhibitors of two classes, the catalytic-domain inhibitors K252a (Kase *et al.*, 1987) and staurosporine (Tamaoki *et al.*, 1986), and sphingosine, a regulatory-domain inhibitor (Hannun *et al.*, 1986), both cause dramatic over-production of DAG second messengers over the concentration range required for inhibition of other PKC actions.

Interpretation of these results is complicated by previous demonstrations that DAG formation also occurs by degradation of phosphatidylcholine (PC) in agonist-stimulated cells. PC hydrolysis to DAG may occur by two mechanisms. Evidence for a PLC mechanism (generating DAG and phosphocholine) has been provided in several systems (Daniel *et al.*, 1986; Besterman *et al.*, 1986; Irving & Exton, 1987; Muir & Murray, 1987; Takuwa *et al.*, 1987; Slivka *et al.*, 1988). Alternatively, PC hydrolysis can occur by the action of phospholipase D, generating choline and phosphatidic acid (PA). PA is subsequently hydrolysed by PA phosphohydrolase, yielding DAG (Bocckino *et al.*, 1987; Pai *et al.*, 1988a,b; Billah *et al.*, 1989b). The mechanism by which PC hydrolysis is initiated in these various systems remains to be established; however, PC hydrolysis by either pathway can be initiated by phorbol ester treatment (see e.g. Daniel *et al.*, 1986; Billah *et al.*, 1989a).

To clarify the interpretation of our data, it was necessary to define the contribution of phosphoinositide and PC turnover to total DAG formation under our experimental conditions. Several approaches were taken, including: (i) examination of the effects of neomycin, a compound reported to inhibit PLC by interaction with the inositol phospholipid substrate (e.g. Slivka & Insel, 1988), and of propranolol, an inhibitor of PA phosphohydrolase (e.g. Abdel-Latif & Smith, 1976; Eichberg *et al.*, 1979); (ii) examination of the effects of phorbol ester treatment; and (iii) direct examination of the turnover of radiolabelled PC. The results are consistent with phosphoinositide turnover being the primary source of DAG in stimulated platelets.

EXPERIMENTAL

Materials

K252a and staurosporine were purchased from Kyowa Hakko U.S.A. Inc. (New York, NY, U.S.A.). D-erythro-Sphingosine, prostaglandin I₂, thrombin (from human plasma), neomycin sulphate, DL-propranolol and PMA were from Sigma. The DAG kinase inhibitor R59022 was from Calbiochem. sn-1,2-Dioctanoylglycerol (diC₈) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL, U.S.A.).

Escherichia coli DAG kinase was prepared as described previously (Preiss *et al.*, 1986) or purchased from Lipidex Inc. (Westfield, NJ, U.S.A.). All radioisotopes were from New England Nuclear.

Preparation and labelling of human platelets

Human platelets were prepared from freshly drawn blood essentially as described by Siess *et al.* (1983). Platelets were resuspended at 6.25×10^8 /ml in modified Tyrode-Hepes medium (134 mM-NaCl, 12 mM-NaHCO₃, 2.9 mM-KCl, 0.34 mM-Na₂HPO₄, 1 mM-MgCl₂, 5 mM-Hepes, 5 mM-glucose, 1 mM-EGTA, pH 7.4).

For experiments where protein phosphorylation or [³²P]PA formation was examined, platelets were labelled for 75 min (at 37 °C) with 0.3 mCi of [³²P]P_i (8500–9120 Ci/mmol)/ml. Platelets were then pelleted (600 g, 10 min) and resuspended in the original volume of Tyrode-Hepes buffer.

For measurement of 5-hydroxytryptamine (serotonin) se-

cretion, 1 μCi of 5-hydroxy[1,2-³H(n)]tryptamine binoxalate (15–30 Ci/mmol)/ml was added to platelet-rich plasma. After a 45 min labelling period, platelets were prepared as described above.

Platelet treatments

Platelets were incubated with the indicated reagents before thrombin stimulation. K252a, staurosporine, PMA and propranolol were added from dimethyl sulphoxide (DMSO) stocks. Sphingosine and the DAG kinase inhibitor R59022 were added as stock solutions in ethanol. The final solvent concentration was 0.5%. In all cases, control platelets were treated with vehicle alone. Neomycin sulphate stock solutions were prepared in Tyrode-Hepes buffer.

Quantification of DAG mass

Unlabelled platelets (6.25×10^8 /ml) were pretreated as described in the Results section before stimulation with a maximal dose of thrombin (1 unit/ml). For measurement of platelet DAG mass, a 0.8 ml sample containing 5×10^8 platelets was extracted at the indicated time, and DAG present in the final chloroform phase was measured by using *E. coli* DAG kinase essentially as described previously (Bishop *et al.*, 1986; Preiss *et al.*, 1986) with minor modifications. Platelet lipid extracts were dried and solubilized in 20 μl of 7.5% β-octylglucoside (Calbiochem) containing 25 mM-dioleoylphosphatidylglycerol (Avanti Polar Lipids). In addition to the detergent/lipid mixed micelles, the final DAG kinase reaction mixture contained: 50 mM-imidazole, pH 6.6, 50 mM-LiCl, 12.5 mM-MgCl₂, 1 mM-EGTA, 2 mM-dithiothreitol, 1 mM-[³²P]ATP and 5 μg of *E. coli* membrane protein in a volume of 100 μl. Conversion of DAG into [³²P]PA was assessed as described by Preiss *et al.* (1986). Data are presented as means ± S.E.M. of multiple determinations. In all cases, similar results were obtained in at least two independent experiments.

Control experiments demonstrated that all inhibitors used for platelet pretreatments do not significantly inhibit the *E. coli* DAG kinase (results not shown). Inhibitors were used at the maximum concentration that they would achieve in the DAG kinase reaction assuming 100% recovery in the platelet lipid extracts: 1.2 μM-PMA, 50 μM-K252a and -staurosporine, 400 μM-propranolol, 1 mM-sphingosine and 45 mM-neomycin sulphate.

PA formation

³²P-labelled platelets were subjected to various pre-treatments as described in the Results section. When phosphorylation of diC₈ was measured, 25 μM-diC₈ (in ethanol) was added immediately before thrombin stimulation. Samples (0.1 ml) containing 6.25×10^7 platelets were extracted at the indicated time, and long-chain endogenous [³²P]PA and [³²P]diC₈ PA were separated by t.l.c. as described previously (Bishop *et al.*, 1986).

5-Hydroxytryptamine secretion

Platelets labelled with 5-hydroxy[³H]tryptamine were pre-incubated with K252a for 1 min before stimulation with 1 unit of thrombin/ml. Secretion was measured at 1 min after addition of thrombin, essentially as described by Tohmatsu *et al.* (1987). Under these conditions secretion of ³H label is maximal. In a typical experiment $88.0 \pm 1.0\%$ of the total ³H label was released in the presence of thrombin. In the absence of thrombin $23 \pm 3\%$ of the label was extracellular.

Protein phosphorylation

[³²P]P_i-labelled platelets were preincubated for 1 min with the indicated concentration of K252a before stimulation with thrombin. Platelet proteins were separated by electrophoresis in

SDS/10%-polyacrylamide gels. Gels were fixed, dried and autoradiographed as previously described (Bishop *et al.*, 1986). The region of the gel containing the 40 kDa PKC substrate was excised and subjected to liquid-scintillation counting. In a typical experiment the 40 kDa region contained 162 ± 31 c.p.m. without thrombin stimulation and 538 ± 41 c.p.m. at 1 min after thrombin addition.

PC labelling and turnover experiments

For [^3H]choline labelling, platelets were resuspended to 5×10^8 /ml in Tyrode's buffer, and $20 \mu\text{Ci}$ of [$\text{methyl-}^3\text{H}$]choline chloride (76 Ci/mmol)/ml was added. After incubation at 37°C for 3 h, platelets were collected by centrifugation, washed once in buffer and resuspended at 5×10^8 /ml. Samples (0.4 ml) were taken before and after stimulation with thrombin or PMA, transferred to 3 ml of chloroform/methanol (1:2, v/v) and extracted by the method of Bligh & Dyer (1956). Portions of both the lower chloroform phase and the aqueous phase were taken for scintillation counting. In some experiments, platelets were pelleted by centrifugation at $10\,000 g$ for 40 s to allow analysis of extracellular radiolabelled choline metabolites. In a representative experiment, a 0.4 ml sample contained approx. 1.1×10^6 total ^3H c.p.m. Approx. 2.3×10^5 c.p.m. were incorporated into chloroform-extractable material, the remainder being water-soluble. Of the water-soluble choline label, over 90% was cell-associated, with approx. 60 000 c.p.m. being extracellular.

For [^3H]myristic acid labelling, platelets were resuspended to 3×10^9 /ml in Tyrode's buffer, incubated with 0.1 mCi of [^3H]myristic acid (52.9 Ci/mmol)/ml for 4 h at 37°C and washed. Some 22% of the cell-associated label was in PC, less than 2% was in other phospholipid species, and the remainder was recovered as non-esterified fatty acid. Platelets were stimulated with thrombin (1 unit/ml) or PMA (100 nM) alone or in the presence of 0.5% ethanol. Samples (0.5 ml) containing 3×10^8 platelets (approx. 50 000 c.p.m. of [^3H]PC) were extracted, and the chloroform phase was analysed by t.l.c. For separation of PA and phosphatidylethanol, the solvent system used was the organic phase from a mixture of ethyl acetate/iso-octane/water/acetic acid (11:5:10:2, by vol).

RESULTS

Phorbol ester pretreatment blocks thrombin-stimulated DAG formation

Upon stimulation of platelets with a maximal dose of thrombin, DAG formation occurs rapidly, reaching a peak at 3.5 times the resting DAG mass level at 0.5 min, then rapidly returning to basal levels by 5 min after thrombin addition (Fig. 1). Other biochemical and physiological responses of platelets (e.g. phosphorylation of the 40 kDa PKC substrate and 5-hydroxytryptamine secretion) are also maximal by 0.5–1 min after stimulation with this dose of thrombin.

Activation of platelet PKC with PMA does not affect platelet DAG mass levels (Fig. 1). No increase in DAG mass was seen for 30 min after addition of PMA from 50 to 500 nM.

Pretreatment of platelets with phorbol ester for 5 min abolishes the thrombin-induced rise in DAG mass (Fig. 1). The PMA dose/response curve is shown in Fig. 2. Maximal inhibition of DAG formation occurs after a 5 min treatment with 250 nM-PMA. Half-maximal inhibition was observed at approx. 60 nM-PMA, in agreement with the half-maximal concentration required for inhibition of InsP_3 formation (Rittenhouse & Sasson, 1985). These results are consistent with previous reports that activation of PKC by phorbol esters uncouples phosphoinositide hydrolysis from agonist receptors.

In several experiments, a late secondary rise in DAG mass was

Fig. 1. Effect of phorbol esters on platelet DAG formation

Platelets were treated with thrombin alone (1 unit/ml; ●), with PMA alone (200 nM; ○), or for 5 min with PMA (200 nM) before thrombin stimulation (1 unit/ml) (▲). At the indicated times after addition of thrombin or PMA, platelet lipids were extracted and DAG was measured as described in the Experimental section. Data are expressed relative to the basal DAG level in unstimulated platelets, and are presented as means $\pm$ S.E.M. for five independent determinations.

Fig. 2. Dose/response for phorbol ester inhibition of thrombin-stimulated DAG formation

Platelets were pretreated with the indicated concentration of PMA for 5 min before stimulation with thrombin (1 unit/ml). Platelet DAG was extracted at 0.5 min after thrombin stimulation. ●, Control; ○, thrombin-stimulated.

observed between 10 and 30 min after thrombin addition, accounting for the larger S.E.M. values observed at these time points in Fig. 1. Since physiological platelet responses occur during the first 1 min, the significance of this rise is not clear. This secondary rise was abolished by PMA pretreatment, suggesting that it is also derived from phosphoinositide hydrolysis; however, the lack of reproducibility of this observation precluded further analysis.

Fig. 3. Inhibition of platelet PKC by K252a

Platelets were pretreated with the indicated concentration of K252a for 1 min before thrombin stimulation (1 unit/ml). Phosphorylation of the 40 kDa substrate (●) was measured at 1 min after thrombin addition to [32 P]P_i-labelled platelets. 5-Hydroxytryptamine secretion (○) was measured at 1 min after addition of thrombin to 5-hydroxy[3 H]tryptamine-labelled platelets. Data are presented relative to the extent of phosphorylation or secretion in platelets not treated with K252a, and represent means $\pm$ S.E.M. for two independent experiments.

K252a and staurosporine potentiate thrombin-stimulated DAG formation

To test further the hypothesis that PKC activation limits the extent of phosphoinositide hydrolysis, we examined the effects of PKC inhibitors on DAG levels in thrombin-stimulated platelets. If negative feedback by PKC is physiologically important, inhibition of PKC should cause over-production of the products of phosphoinositide hydrolysis. The first inhibitor examined was K252a, a potent PKC inhibitor (K_i 25 nM *in vitro*), which acts by competing with ATP for binding to the catalytic domain (Kase *et al.*, 1987).

PKC activation is thought to be an essential step in the signal-transduction pathway leading to the physiological responses of platelets (see, e.g., Hannun *et al.*, 1987). As reported previously (Yamada *et al.*, 1987), inhibition of PKC by K252a blocks phosphorylation of the 40 kDa PKC substrate [IC_{50} (concn. giving 50% inhibition) = 2.1 μM] and prevents 5-hydroxytryptamine secretion (IC_{50} = 1.2 μM) in thrombin-stimulated platelets (Fig. 3). Inhibition of secretion is complete after a 1 min preincubation with 10 μM-K252a.

When platelets are pretreated with 10 μM-K252a for 1 min before thrombin stimulation, a much greater DAG response is observed (Fig. 4). In control platelets, thrombin induces a 3.5-fold increase in DAG levels (from 36.2 to a maximum of 122.8 pmol/ 5×10^8 platelets at 0.5 min in one experiment). In the presence of 10 μM-K252a, maximal DAG levels achieved were 10-fold higher than resting levels (361.7 pmol/ 5×10^8 platelets) (Fig. 4). K252a does not significantly alter basal DAG levels or the time course of DAG formation and attenuation.

The effect of K252a on thrombin-stimulated DAG levels is observed over the same concentration range needed to inhibit 5-hydroxytryptamine secretion (Table 1). Similar results were obtained with a related protein kinase inhibitor, staurosporine (Table 1).

K252a does not inhibit [32 P]PA formation

This effect of K252a on DAG levels could be attributed to increased DAG formation (i.e. enhanced phosphoinositide

Fig. 4. K252a potentiates thrombin-induced DAG formation

Platelets were pretreated for 1 min with 10 μM-K252a (○) or with 0.5% DMSO (●, control) before stimulation with 1 unit of thrombin/ml. At various times after thrombin addition, platelet DAG was extracted. The data are expressed relative to DAG levels in unstimulated control platelets, and represent means $\pm$ S.E.M. for two independent experiments.

Table 1. Dose-dependence for K252a and staurosporine potentiation of DAG formation

Platelets were pretreated with the indicated concentration of K252a or staurosporine (Stauro) or with DMSO (0.5%) for 1 min. Platelet DAG was extracted either before thrombin stimulation (–) or 0.5 min after the addition of 1 unit of thrombin/ml (+). Data represent means $\pm$ S.E.M. of duplicate determinations.

Pretreatment	Thrombin	DAG	
		(pmol/ 5×10^8 platelets)	(% of control response)
DMSO	–	33.4	–
10.0 μM-K252a	–	32.5	–
DMSO	+	118.8 $\pm$ 4.4	(100)
0.1 μM-K252a	+	133.8	117.6
0.5 μM-K252a	+	151.2 $\pm$ 12.0	137.9
1.0 μM-K252a	+	175.7 $\pm$ 0.9	205.7
5.0 μM-K252a	+	267.4 $\pm$ 1.0	313.1
10.0 μM-K252a	+	393.2 $\pm$ 5.0	421.3
DMSO	–	38.4	–
10.0 μM-Stauro	–	36.5	–
DMSO	+	120.9	(100)
1.0 μM-Stauro	+	188.2	181.6
5.0 μM-Stauro	+	235.2	285.1
10.0 μM-Stauro	+	267.9	324.7

hydrolysis) or decreased DAG metabolism. DAG metabolism occurs primarily by phosphorylation to PA catalysed by DAG kinase (Bishop & Bell, 1986).

To rule out an effect of K252a on DAG phosphorylation, we measured the rate of 32 P labelling of PA after thrombin stimulation. Pretreatment of platelets for 1 min with 10 μM-K252a before thrombin stimulation enhanced incorporation of 32 P into PA approx. 2-fold (Fig. 5). This result is consistent with an increased flux through DAG and provides evidence that DAG kinase is not inhibited.

The ability of platelet DAG kinase to phosphorylate an exogenous cell-permeant DAG, diC₈, was also examined. The rate of diC₈ phosphorylation is unaffected by prior treatment with 10 μM-K252a (Fig. 6a). Staurosporine (10 μM) also did not

Fig. 5. K252a increases formation of $[^{32}\text{P}]\text{PA}$ in thrombin-stimulated platelets

$[^{32}\text{P}]\text{P}_i$ -labelled platelets were preincubated for 1 min in the presence of $10\text{ }\mu\text{M}$ -K252a (○) or 0.5% DMSO (●, control) before stimulation with 1 unit of thrombin/ml. At the indicated times, platelet lipids were extracted and analysed for ^{32}P incorporation into PA.

affect this rate (results not shown). In contrast, pretreatment with $50\text{ }\mu\text{M}$ -R59022, a known DAG kinase inhibitor (de Chaffoy de Courcelles *et al.*, 1985), decreases the rate of diC_8 phosphorylation by approx. 50%.

A similar experiment was also performed in thrombin-stimulated platelets (Fig. 6b). Thrombin stimulation does not alter the rate of diC_8 phosphorylation. However, $10\text{ }\mu\text{M}$ -K252a slows the rate of diC_8 phosphorylation in stimulated platelets. A more pronounced effect on diC_8 phosphorylation was observed with R59022. The decreased rate of diC_8 phosphorylation in the presence of K252a may be due to elevated endogenous DAG competing with diC_8 for DAG kinase (e.g. MacDonald *et al.*, 1988a,b).

Sphingosine potentiates thrombin-stimulated DAG formation

The above results suggest that DAG over-production in the presence of K252a was due to enhanced DAG formation and not to a decreased rate of DAG metabolism. To support this further, we examined the effect of another PKC inhibitor, sphingosine, on DAG formation. Unlike K252a and staurosporine, sphingosine inhibits PKC by interacting with the regulatory lipid-binding domain (Hannun *et al.*, 1986); therefore interaction of sphingosine with the ATP site of DAG kinase would be unlikely.

Pretreatment of platelets for 1 min with concentrations of sphingosine required to inhibit PKC-mediated events in response to thrombin (i.e. $10\text{--}25\text{ }\mu\text{M}$) (Hannun *et al.*, 1986, 1987) results in enhanced DAG formation upon thrombin stimulation (Fig. 7). In the experiment shown in Fig. 7, peak DAG levels achieved in response to thrombin were $118\text{ pmol}/5 \times 10^8$ platelets in control platelets, 206 in the presence of $12.5\text{ }\mu\text{M}$ -sphingosine and 275 in the presence of $25\text{ }\mu\text{M}$ -sphingosine.

Similar results were seen with $50\text{ }\mu\text{M}$ -sphingosine; however, higher concentrations result in less DAG over-production (Table 2). Thrombin-stimulated DAG levels reached a peak at $150\text{ pmol}/5 \times 10^8$ cells with $100\text{ }\mu\text{M}$ -sphingosine; $200\text{ }\mu\text{M}$ -sphingosine nearly abolished thrombin-stimulated DAG formation, and slightly decreased basal (resting) DAG levels (Table 2). These higher concentrations of sphingosine may begin to disrupt the coupling of thrombin receptors to PLC activation, possibly owing to lytic or cytotoxic effects of this compound when added in higher concentrations in ethanol or DMSO (Pittet *et al.*, 1987; Lambeth *et al.*, 1988).

Fig. 6. Effect of K252a and R59022 on phosphorylation of an exogenous cell-permeant DAG

(a) Platelets were pretreated for 1 min with 0.5% DMSO (●, control), $10\text{ }\mu\text{M}$ -K252a (○) or $50\text{ }\mu\text{M}$ -R59022 (▲) before addition of diC_8 ($25\text{ }\mu\text{M}$). At the indicated times, platelet lipids were extracted and the formation of $[^{32}\text{P}]\text{diC}_8\text{ PA}$ was measured. (b) This experiment was performed as described for (a), except that platelets were stimulated with thrombin (1 unit/ml) immediately after addition of diC_8 . Data represent means $\pm$ S.E.M. of duplicate determinations.

PC hydrolysis is not detectable upon thrombin or phorbol ester treatment

The above results are consistent with a role for PKC in limiting phosphoinositide hydrolysis in thrombin-stimulated platelets. However, it is clear that in many cell types hydrolysis of other phospholipids (primarily PC) contributes to DAG production. Two approaches were used to examine the possible contribution of PC hydrolysis under our experimental conditions.

First, cellular PC pools were prelabelled by several methods. Attempts to label platelet PC by preincubation with $[^3\text{H}]1\text{-O-alkyl-2-lyso-PC}$ (Pai *et al.*, 1988a,b) were unsuccessful. This compound became cell-associated, but was not converted into $[^3\text{H}]1\text{-O-alkyl-2-acyl-PC}$, perhaps owing to low lyso-PC acyltransferase activity in platelets. However, efficient labelling was achieved with $[^3\text{H}]\text{myristate}$. Under the labelling conditions employed, $[^3\text{H}]\text{myristate}$ selectively labelled platelet PC (but not phosphoinositides), to a specific radioactivity of approx. $1400\text{ c.p.m.}/\text{nmol}$ (based on 120 nmol of PC/ 10^9 platelets; Takamura *et al.*, 1987). Upon stimulation with thrombin or phorbol ester, no incorporation of $[^3\text{H}]\text{myristate}$ into PA, DAG or phosphatidylethanol (when reactions were run in the presence of ethanol) was observed (results not shown). In $^3\text{H}/^{32}\text{P}$ double-labelling experiments, the same platelets were found to in-

Fig. 7. Sphingosine potentiates thrombin-induced DAG formation

Platelets were pretreated for 1 min with the indicated concentration of sphingosine (○, 12.5 μM; ▲, 25 μM) or with 0.5% ethanol (●, control) before stimulation with 1 unit of thrombin/ml. At the indicated times platelet lipids were extracted and DAG was analysed.

Table 2. Dose-dependence for sphingosine potentiation of DAG formation

Platelets were pretreated with the indicated concentration of sphingosine (Sphingo) for 1 min. Platelet DAG was extracted either before addition of thrombin (–) or 0.5 min after thrombin stimulation (+). Data represent means ± S.E.M. of duplicate determinations.

Pretreatment	Thrombin	DAG	
		(pmol/5 × 10 ⁸ platelets)	(% of control response)
None	–	31.9	–
200 μM-Sphingo	–	21.1	–
None	+	132.9 ± 1.3	(100)
10 μM-Sphingo	+	214.0 ± 36	180.3
50 μM-Sphingo	+	274.0 ± 52	239.7
100 μM-Sphingo	+	151.0 ± 12	117.9
200 μM-Sphingo	+	43.4 ± 5.6	11.4

corporate [³²P]P_i into PA by the DAG kinase pathway. Similar results were obtained in the presence of K252a.

Platelet PC pools were also labelled by preincubation with [³H]choline, and release of intra- and extra-cellular water-soluble choline metabolites was measured. Treatment with thrombin or PMA in the presence or absence of PKC inhibitors did not stimulate release of water-soluble [³H]choline label from platelets (results not shown).

The effects of available inhibitors of phospholipid hydrolytic pathways were also examined. Neomycin has been reported to inhibit phosphoinositide hydrolysis in thrombin-stimulated plate-

Table 3. Effect of neomycin and propranolol on DAG formation

Platelets were pretreated with the indicated concentration of neomycin sulphate (Neo) or propranolol (Pro) for 5 min and extracted immediately (–) or after stimulation for 0.5 min with 1 unit of thrombin/ml (+). The effects of neomycin or propranolol are expressed relative to the response observed in the absence of drug after subtracting the basal DAG level. Neomycin data are means ± S.E.M. from two experiments, performed in duplicate; propranolol data are representative of two similar experiments.

Pretreatment	Thrombin	DAG	
		(pmol/5 × 10 ⁸ platelets)	(% of control response)
None	–	33.1 ± 3.8	–
10.0 mM-Neo	–	36.9	–
None	+	147.5 ± 7.1	(100)
2.5 mM-Neo	+	82.7 ± 1.1	43.3
5.0 mM-Neo	+	63.0 ± 2.5	26.1
10.0 mM-Neo	+	41.7 ± 6.9	7.5
None	–	29.7	–
200 μM-Pro	–	21.9	–
None	+	140.4	(100)
100 μM-Pro	+	166.1	123.2
200 μM-Pro	+	230.1	181.0

lets. Neomycin sulphate completely inhibits the thrombin-induced rise in DAG mass (IC₅₀ = 2.8 mM) (Table 3). Maximal inhibition was achieved after a 5 min preincubation with 10 mM-neomycin. This is the same concentration range required to prevent phosphoinositide hydrolysis (Tysnes *et al.*, 1988), and is consistent with phosphoinositide hydrolysis being the primary source of DAG in these experiments. In control experiments, where 10 mM-neomycin sulphate completely inhibited the thrombin response, 30 mM-Na₂SO₄ decreased the response by 34%. This suggests that, although some of the decreased DAG response may be due to the high concentrations of sulphate present, most of the inhibition is mediated by neomycin.

Propranolol has been reported to inhibit PA phosphohydrolase; therefore this compound should prevent DAG formation by the phospholipase D pathway. Pretreatment of platelets with propranolol at concentrations up to 200 μM does not inhibit DAG formation induced by thrombin (Table 3). In fact, treatment with 200 μM-propranolol enhances DAG formation (1.6–2.7-fold) after thrombin stimulation, whereas propranolol alone does not elevate platelet DAG levels. The mechanism of the potentiating effect of propranolol requires further investigation.

DISCUSSION

Phosphoinositide turnover is the primary source of DAG in thrombin-stimulated platelets

To understand the observed effects of PKC inhibitors on DAG mass levels, it was necessary to examine the pathways by which activated platelets generate DAG. Several lines of evidence indicate that phosphoinositide hydrolysis is the predominant source of DAG under the conditions used.

(i) Activation of PKC by phorbol esters induces PC hydrolysis (e.g. Billah *et al.*, 1989a) and subsequently DAG formation (e.g. Rider *et al.*, 1988) in a number of cell types. In contrast, phorbol ester treatment blocks agonist-stimulated phosphoinositide hydrolysis. In human platelets, phorbol esters alone did not induce DAG formation. In neutrophils, where PMA activates

PC-hydrolytic pathways, DAG mass formation is observed by 1 min and is maximal by about 30 min after PMA addition (Rider *et al.*, 1988). In addition, pretreatment of platelets with PMA blocked subsequent thrombin-stimulated DAG formation.

(ii) The putative PLC inhibitor neomycin abolishes thrombin-induced DAG formation over the concentration range needed to suppress platelet phosphoinositide turnover and aggregation in response to thrombin (Tysnes *et al.*, 1988). Neomycin appears to inhibit PLC activity by interacting with the phosphoinositide substrate (Lipsky & Leitman, 1982). Although neomycin may not be a specific inhibitor of PLC activity, it has been demonstrated to be an effective inhibitor of phosphoinositide turnover (Tysnes *et al.*, 1987) and not to inhibit phospholipid degradation by a phospholipase A2 mechanism (Slivka & Insel, 1988) or PC hydrolysis in dog kidney cells stimulated with α_1 -adrenergic agents (Slivka *et al.*, 1988). In contrast, the PA phosphohydrolase inhibitor propranolol does not inhibit thrombin-induced DAG formation. Propranolol abolishes DAG formation via the phospholipase D pathway in neutrophils stimulated with chemotactic peptide (Billah *et al.*, 1989b).

(iii) Finally, direct attempts to examine PC turnover by radiolabelling failed to detect formation of PA or phosphatidylethanol (a marker for phospholipase D activation) from PC, or release of choline metabolites. In neutrophils and HL60 granulocytes, 4% and 10% of the cellular PC turns over in response to chemotactic peptide (Billah *et al.*, 1989b) or PMA stimulation (Billah *et al.*, 1989a) respectively. This extent of PC hydrolysis should have been detected by the labelling strategies employed.

The conclusion that phosphoinositide hydrolysis is the primary source of agonist-stimulated DAG in platelets is consistent with previous observations. Broekman *et al.* (1981) found that PA formed in stimulated platelets has a fatty acid composition similar to that of the phosphoinositides, enriched in stearic and arachidonic acid. In addition, Muir & Murray (1988) found that phorbol esters (and thrombin) do not stimulate phosphate incorporation into PC in platelets (as they do in cell types where PC degradation is stimulated).

In contrast, Rubin (1988) observed the formation of phosphatidylethanol in thrombin-stimulated platelets prelabelled with [3 H]arachidonic acid. This was attributed to trans-phosphatidyl transfer catalysed by phospholipase D in the presence of ethanol. No quantitative results were provided. It is possible that our failure to detect phosphatidylethanol formation in response to thrombin was due to the inclusion of 1 mM-EGTA in the reaction buffer. However, we observed that the amount of DAG generated in response to thrombin was similar in the presence or absence of 1 mM exogenous Ca^{2+} (W. R. Bishop, unpublished work). Our results suggest that, if phospholipase D is present in platelets, it does not represent a major pathway for DAG formation under our experimental conditions.

PKC plays a negative-feedback role in thrombin-stimulated platelets

The finding that phorbol ester pretreatment abolished DAG formation in stimulated platelets, and the previous reports of the inhibitory effects of phorbol esters on phosphoinositide turnover, have led to the hypothesis that PKC activation shuts off phosphoinositide hydrolysis in stimulated cells. If PKC plays a negative-feedback role in agonist-stimulated cells, then treatment of cells with inhibitors of PKC should prevent this feedback, resulting in over-production of the PLC products. The results reported here provide strong support for a physiological role of PKC in suppressing phosphoinositide hydrolysis after thrombin stimulation. Pretreatment of platelets with two distinct classes of

PKC inhibitors resulted in a 2–4-fold over-production of DAG second messengers.

In contrast, Watson *et al.* (1988) observed no effect of staurosporine on inositol phosphate formation or on the formation of [32 P]PA in thrombin-stimulated platelets. They concluded that the inhibition of PLC activity observed in cells treated with phorbol esters or cell-permeable DAG is not of physiological significance. Their results contrast with those reported here. We observed increased formation both of DAG mass and of [32 P]PA in platelets treated with K252a. Since both DAG and InsP_3 second messengers are rapidly metabolized, the ability to observe second-messenger over-production will depend on the time points chosen. Watson *et al.* (1988) measured inositol phosphate levels at 1 and 10 min after thrombin stimulation, and could have missed a transient over-production.

Crouch & Lapetina (1989) reported that preincubation of platelets with 1 μM -staurosporine enhances the initial formation of [3 H]inositol-labelled InsP_2 and InsP_3 in thrombin-stimulated cells. King & Rittenhouse (1989) also observed increased inositol polyphosphate accumulation in staurosporine-treated platelets stimulated with thrombin for short time periods (10 s). They attributed this increase to blockade of PKC-mediated stimulation of InsP_3 metabolism. Our finding that the lipid products of phosphoinositide hydrolysis are also over-produced argues strongly that PKC inhibitors also act to prevent negative-feedback regulation of this hydrolysis.

The effect of K252a and staurosporine on platelet DAG levels does not appear to be due to inhibition of platelet DAG kinase. First, the time course of DAG formation and attenuation is similar in the presence or absence of PKC inhibitors. If platelets are treated with 1-mono-oleoylglycerol, an inhibitor of DAG kinase, DAG levels remain elevated after stimulation (Bishop *et al.*, 1986). Secondly, we found that K252a does not inhibit, but rather enhances, [32 P]PA formation from endogenous DAG in thrombin-stimulated platelets. This is consistent with increased DAG formation leading to increased substrate availability for DAG kinase. Finally, in agreement with the findings of Watson *et al.* (1988), we found that K252a and staurosporine do not inhibit phosphorylation of diC_8 by unstimulated platelets. Surprisingly, K252a did decrease the rate of diC_8 phosphorylation in thrombin-stimulated platelets. Since the rate of diC_8 phosphorylation is similar in control and thrombin-stimulated platelets, this does not appear to be due to preventing PKC-mediated activation of DAG kinase. It is possible that the slower rate of diC_8 phosphorylation in K252a-treated cells is due to the large increase in endogenous DAG formed in these cells acting as a competitive inhibitor with respect to diC_8 . MacDonald *et al.* (1988a) found that the rate of phosphorylation of a cell-permeant saturated DAG (didecanoylglycerol; diC_{10}) was decreased in platelet-derived-growth-factor-stimulated fibroblasts, owing to competition by the expanded pool of endogenous DAG (MacDonald *et al.*, 1988a,b).

In the present studies we have not ruled out a potential effect of PKC inhibitors on the DAG lipase pathway. Inhibition of DAG lipase is less likely to contribute to the observed DAG over-production, since this is a relatively minor pathway compared with DAG kinase (Bishop & Bell, 1986). In addition, K252a and staurosporine are more likely to inhibit DAG kinase than DAG lipase, owing to the known mechanism of action of these compounds (e.g. Kase *et al.*, 1987).

Desensitization of thrombin activation of PLC

The mechanism by which PKC mediates its inhibitory effects on phosphoinositide hydrolysis has not been established. Negative feedback may occur by disruption of the coupling of PLC to

the G-protein, perhaps by phosphorylation of a PLC isoenzyme or of the G-protein.

Considerable progress has been made in the purification and cloning of multiple forms of phosphoinositide-specific PLC (reviewed by Rhee *et al.*, 1989). Multiple forms of PLC are found in human platelets; their relation to the cloned forms remains to be resolved (Banno *et al.*, 1988). A PLC of 143 kDa was identified in platelet cytosol (Hakata *et al.*, 1982), but appears to be immunologically distinct from the bovine brain forms of similar mass (Ryu *et al.*, 1987). Two forms of PLC have been resolved from human platelet membranes; one of these was identified as a 61 kDa protein (Banno *et al.*, 1988). The relationship of this protein to a 62 kDa form of the enzyme purified and cloned from guinea-pig uterus (Bennett & Crooke, 1987; Bennett *et al.*, 1988) also remains to be determined. The latter enzyme is phosphorylated upon stimulation of rat basophilic leukaemia cells with phorbol esters, and is a substrate for PKC *in vitro* (Bennett & Crooke, 1987). The effect of phosphorylation on enzymic activity has not been reported.

Desensitization may also occur by PKC-mediated phosphorylation of the coupling G-protein. The nature of the G-protein involved in coupling cell-surface receptors to PLC activation has not been elucidated. Previous work suggests that either G_i (Crouch & Lapetina, 1988) or a novel G protein, G_z (Carlson *et al.*, 1989), may play an important role in thrombin-induced activation of PLC in platelets and that the α subunit of these G-proteins is phosphorylated in stimulated platelets.

Despite prevention of the negative-feedback role of PKC on phosphoinositide hydrolysis by using PKC inhibitors, DAG formation still reaches a peak at 0.5 min after stimulation and rapidly returns to basal levels. This indicates that additional mechanisms exist to desensitize thrombin-induced phosphoinositide hydrolysis. Crouch & Lapetina (1989) have provided evidence that platelet agonist receptors desensitize by two distinct mechanisms, one of which is independent of PKC. Paris *et al.* (1988) have provided evidence for PKC-independent homologous desensitization of thrombin receptors in hamster lung fibroblasts which appears to result from modification of the thrombin receptor, uncoupling it from its G-protein.

The potentiating effect of PKC inhibitors on phosphoinositide hydrolysis and second-messenger formation may have important consequences for the use of PKC inhibitors in intact cells. First, enhanced phosphoinositide hydrolysis may increase InsP₃ levels and enhance the extent and duration of the Ca²⁺ signal (Crouch & Lapetina, 1988, 1989). Secondly, over-production of DAG may modulate the activity of other membrane-associated enzymes. Candidates for DAG-modulated enzymes include phospholipase A₂ (e.g. Kolesnick & Paley, 1987) and sphingomyelinase (Kolesnick, 1987). DAG over-production also increases the available substrate for DAG lipase, an enzyme implicated in mobilization of arachidonic acid (Bell *et al.*, 1979). Therefore potentiation of the DAG signal may cause perturbations in pathways of lipid metabolism and signal transduction.

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