Platelet phospholipase D is activated by protein kinase C via an integrin $\alpha_{IIb}\beta_3$-independent mechanism

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Blood platelets contain phospholipase D (PLD) that is rapidly activated following platelet stimulation. It is currently unclear, however, where PLD fits into the signalling cascade that leads to aggregation and secretion. Therefore we investigated the mechanism of activation of PLD in human platelets, using the formation of the PLD-specific product phosphatidylethanol as a measure of PLD activity. PLD was activated by a number of platelet agonists that also cause the activation of protein kinase C, including thrombin, collagen, the Ca^{2+} ionophore A23187 and the thromboxane A_2-mimetic U46619. Phorbol 12-myristate 13-acetate (PMA), a direct activator of protein kinase C, also increased PLD activity. A selective inhibitor of protein kinase C, Ro-31-8220, totally blocked the stimulation of PLD by thrombin or PMA under conditions in which it also inhibited phosphorylation of pleckstrin, the major protein kinase C substrate in platelets. Ro-31-8220 additionally inhibited A23187-stimulated PLD activity, indicating that Ca^{2+} activation of PLD also occurs via a protein kinase C-dependent pathway. In the presence of the fibrinogen antagonist peptide RGDS, which inhibits fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ and allows little or no aggregation to occur, thrombin- and PMA-stimulated PLD activity was still observed, indicating that PLD activation is not simply a consequence of platelet aggregation. Furthermore, these agonists were able to stimulate PLD in platelets from a Glanzmann's thrombasthenia type I patient lacking the integrin $\alpha_{IIb}\beta_3$ complex, which indicates that activation of PLD is also independent of the recruitment of integrin $\alpha_{IIb}\beta_3$. Taken together, our results show that PLD is activated by a pathway involving protein kinase C, and suggest that PLD might be involved in signal transduction events occurring upstream of integrin $\alpha_{IIb}\beta_3$ activation and fibrinogen binding, which are prerequisites for full platelet aggregation.

INTRODUCTION

Phospholipase D (PLD) is rapidly activated in a wide variety of cell types in response to a number of agonists and hormones [1,2]. Hydrolysis of phosphatidylcholine by PLD results in the production of phosphatidic acid (PA), a putative second messenger that has been shown to cause actin polymerization [3] and Ca^{2+} mobilization and mitogenesis in fibroblasts [4]. Deacylation of PA by phospholipase A_2 produces lysoPA, a fairly water-soluble lipid mediator that has been demonstrated to produce a wide array of cellular responses, primarily through G-protein-dependent pathways [5]. Dephosphorylation of PA by a phosphatase produces diacylglycerol, a second messenger that activates protein kinase C. Diacylglycerol species arising from the hydrolysis of phosphatidylcholine are distinct in fatty acid composition and time course of formation from diacylglycerol produced as a result of phosphoinositide hydrolysis by phospholipase C [6]; this could have important consequences for the activation of the numerous isoforms of protein kinase C [7].

Platelets contain PLD that is activated upon platelet stimulation [8–11]. The role of PLD in platelet physiology is currently unknown, but PA, lysoPA or diacylglycerols arising directly or indirectly through the action of PLD might be involved in shape change, aggregation and/or secretion. It has been shown that PA and lysoPA are able to cause platelet aggregation [12], and that lysoPA is released from activated platelets [13]. PA and lysoPA enhance the binding of integrin $\alpha_{IIb}\beta_3$ to its ligand, fibrinogen, which is required for aggregation [14]. Finally, diacylglycerol is produced in multiple phases in thrombin-stimulated platelets [15], which might be a result of the hydrolysis of various phospholipids sequentially by phospholipase C and PLD.

An understanding of the mechanism of activation of PLD in platelets could provide a clue about its potential role in platelet physiology. It has been demonstrated in a number of cell types and tissues that PLD is activated as a consequence of protein kinase C activation [2,16], which is secondary to phosphoinositide hydrolysis by phospholipase C. On the other hand, a number of reports document protein kinase C-independent activation of PLD, and activation has been shown to occur independent of phosphoinositide hydrolysis in certain cell types (reviewed in [2]). Whether protein kinase C plays a role in PLD activation in platelets is equivocal: there is some evidence for the involvement of protein kinase C [10,17] as well as some against [9]. To address this question we used the protein kinase C inhibitor Ro-31-8220, which has been shown to be more selective for protein kinase C than its commonly used parent compound, staurosporine [18].

In platelets, protein kinase C is known to be involved in the pathway leading to the conformational change in integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) that is responsible for the exposure of fibrinogen binding sites [19], which initiates platelet aggregation [20]. However, neither protein kinase C nor any other serine/threonine protein kinase is thought to cause activation of the

Abbreviations used: ACD, acid/citrate/dextrose; 4α-PDD, 4α-phorbol 12,13-didecanoate; PA, phosphatidic acid; PEt, phosphatidylethanol; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate.

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integrin by direct phosphorylation [21]; an intermediary signalling molecule (or molecules) must be involved [22]. Therefore we investigated additionally the position that PLD occupies in this signalling cascade, which might give information about which events are controlled by PLD.

MATERIALS AND METHODS

Preparation of platelets

Suspensions of washed platelets were prepared as described [11,23]. Briefly, blood was drawn from healthy volunteers (who had not taken medication for 2 weeks prior to donation) into 1/6 vol. acid/citrate/dextrose (ACD; 38 mM citrate, 88 mM sodium citrate, 124 mM glucose; Biotrans, Dreieich, Germany) and centrifuged at 120 *g* for 20 min. To the resulting platelet-rich plasma was added (per ml) 111 μl of ACD and 2 units of apyrase (Grade III; Sigma, Munich, Germany), and platelets were collected by centrifugation at 450 *g* for 7 min at room temperature. The platelet pellet was suspended in a modified Tyrode's buffer (buffer A; 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mg/ml glucose, pH 6.4) containing 2.5 units/ml apyrase and 1 unit/ml hirudin (Paesel & Lorei, Frankfurt, Germany), incubated at 37 °C for 15 min and centrifuged again for 7 min at 450 *g*. This washing step was repeated once, and platelets were finally suspended at $(9–12) \times 10^8$/ml in buffer A supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$, pH 7.4 (referred to as buffer B).

Measurement of PLD activity

PLD activity was assessed by measuring the formation of [^{3}H]phosphatidylethanol ([^{3}H]PEt) in cells labelled with [^{3}H]myristic acid, which selectively labels phosphatidylcholine over other phospholipids [16,24,25]. PEt is formed from phospholipids in the presence of ethanol via transphosphatidylation, a reaction that is specific to PLD [26,27]. Washed platelets in buffer B were labelled with 35–50 μCi/ml [^{3}H]myristic acid (34 Ci/mmol; DuPont NEN, Bad Homburg, Germany) at 37 °C for 2–3 h with occasional mixing. The suspension was then further diluted to 3×10^8 platelets/ml in buffer B, and 180 μl of this suspension was treated with various agonists in the presence of 0.5% (v/v) ethanol. Incubations were carried out at 37 °C in a total volume of 200 μl in a 2 ml round-bottom tube containing a stainless steel sphere (2 mm diameter; SKF, Schweinfurt, Germany) in an Eppendorf thermomixer rotating at 750 rev./min. Preincubation of labelled platelets with Ro-31-8220 (a gift from Roche Products Ltd., Welwyn Garden City, Herts., U.K.) or the tetrapeptide RGDS (Sigma) was carried out for 20 min in a thermomixer at 37 °C. Incubation with collagen (Hormon-Chemie, Munich, Germany), bovine thrombin or U46619 was for 10 min, and with A23187, phorbol 12-myristate 13-acetate (PMA) or 4α-phorbol 12,13-didecanoate (4α-PDD) for 20 min (all other compounds from Sigma). To stop incubations, 1 ml of ice-cold methanol was added, lipids were extracted into chloroform, and [^{3}H]PEt was quantified by TLC and scintillation counting as previously described [28]. ^{3}H-labelled products were scraped from portions of thin-layer plates corresponding to unlabelled authentic PEt standard (Biomol, Hamburg, Germany). Background radioactivity co-migrating with PEt standard (observed in the absence of ethanol) was subtracted from ^{3}H radioactivity obtained for incubations containing ethanol. This background radioactivity was equal to that resulting from control incubations performed in the presence of ethanol but without agonists; therefore intact platelets were observed to have no basal PLD activity. Background radioactivity was unaffected by agonist treatments.

Platelet aggregation

After a 2 h incubation of platelets in buffer B, the suspension was diluted to 3×10^8 platelets/ml in buffer B and used for aggregation measurements in an Icare AG12 902/1 aggregometer for 10 or 20 min at 37 °C with stirring at 1500 rev./min, according to the method of Born [29]. Preincubations with the peptide RGDS were performed in a thermomixer rotating at 750 rev./min for 20 min at 37 °C.

RESULTS

Incubation of [^{3}H]myristic-acid-labelled platelets with thrombin resulted in the production of [^{3}H]PEt, the specific product of PLD (Table 1). This response, which was maximal at 0.3 unit/ml thrombin, occurred within 15 s of stimulation and attained a plateau phase after 10 min (results not shown). Other agonists that cause platelet aggregation, such as the phorbol ester PMA, collagen, the thromboxane A_2-mimetic U46619 and the Ca^{2+} ionophore A23187, also promoted the activation of PLD (Table 1), whereas the platelet antagonists sodium nitroprusside and forskolin had no effect (results not shown).

The finding that the direct activation of protein kinase C by PMA increased PLD activity implies that this enzyme might regulate PLD activity during platelet activation. A control experiment showed that the analogue of PMA that does not activate protein kinase C, 4α-PDD, had no effect on PLD activity (Table 1). In order to investigate further the role of protein kinase C in PLD activation, we tested the effects of the selective inhibitor of protein kinase C, Ro-31-8220 [18], on the PLD response. This compound inhibited the maximal formation of [^{3}H]PEt produced by thrombin or PMA in a concentration-dependent manner (Figure 1). We verified that Ro-31-8220 inhibited protein kinase C activity under these conditions by demonstrating that the PMA- and thrombin-dependent phosphorylation of pleckstrin, the major substrate of protein kinase C in platelets [30], was inhibited (results not shown).

Table 1 Effects of platelet activators/inhibitors on PLD activity

Washed platelets were prepared and labelled with [^{3}H]myristic acid, and PLD activity was assessed as described in the Materials and methods section. Platelets were treated with various agents for 10 min (except for PMA, 4α-PDD and A23187; 20 min) in the presence of 0.5% ethanol at the following concentrations: 0.3 unit/ml thrombin, 300 nM PMA, 300 nM 4α-PDD, 100 μg/ml collagen, 1 μM U46619 and 10 μM A23187. PLD activity, determined by the amount of [^{3}H]PEt formed, is expressed as a percentage of [^{3}H]PEt obtained for thrombin treatment in each experiment (set to 100%). A typical value was 480 d.p.m./sample for thrombin treatment (above a background of 100–150 d.p.m.). Data represent means($\pm$S.E.M.) or range for the numbers of independent determinations shown in parentheses. Values indicated by * are significantly different from zero ($P < 0.05$; Student's *t*-test).

Stimulus	PLD activity (%)
Thrombin	100*
PMA	53 $\pm$ 4 (14)*
4α-PDD	3 $\pm$ 1 (2)
Collagen	57 $\pm$ 17 (4)*
U46619	22 $\pm$ 6 (3)*
A23187	34 $\pm$ 8 (4)*

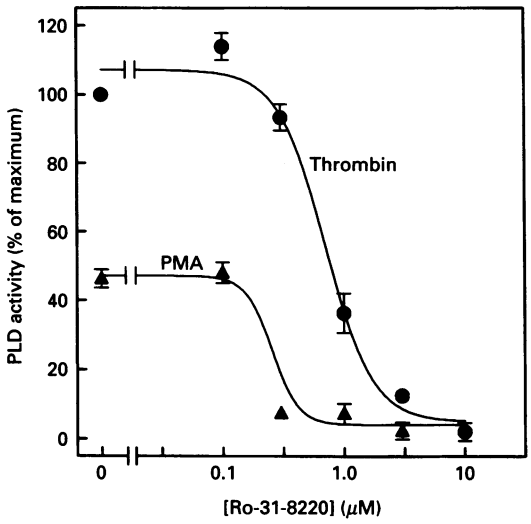

Figure 1 Effect of Ro-31-8220 on thrombin- and PMA-stimulated activity

[^{3}H]Myristic-acid-labelled platelets were treated with the indicated concentrations of Ro-31-8220 (or 0.5% DMSO vehicle) for 20 min at 37 °C, after which thrombin (0.3 unit/ml) or PMA (300 nM) was added for 10 or 20 min respectively in the presence of 0.5% ethanol. PLD activity was determined as described in the Materials and methods section. PLD activity is expressed as a percentage of that obtained with thrombin in the absence of Ro-31-8220. Ro-31-8220 alone (10 μM) had no effect on background (unstimulated) radioactivity recovered. Data are means (±S.E.M.) for triplicate determinations from one experiment that is representative of several. Error bars are not shown for data points having errors that are smaller than the symbol.

The fact that the Ca^{2+} ionophore A23187 activates PLD (Table 1) is consistent with the idea that intracellular Ca^{2+} plays a role in the activation of PLD in platelets, as suggested previously [9]. Since A23187 has been shown to activate protein kinase C in platelets [31], we tested whether the effects of the Ca^{2+} ionophore

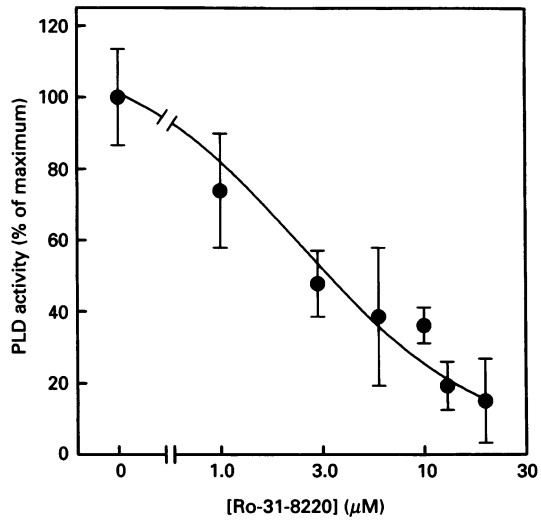

Figure 2 Effect of Ro-31-8220 on A23187-stimulated PLD activity

Platelets labelled with [^{3}H]myristic acid were treated with the given concentrations of Ro-31-8220, as described in the legend to Figure 1, and then challenged with 10 μM A23187 for 20 min. PLD activity is expressed as a percentage of the response obtained with A23187 in the absence of Ro-31-8220. Data shown are means (±S.E.M.) of values from three experiments.

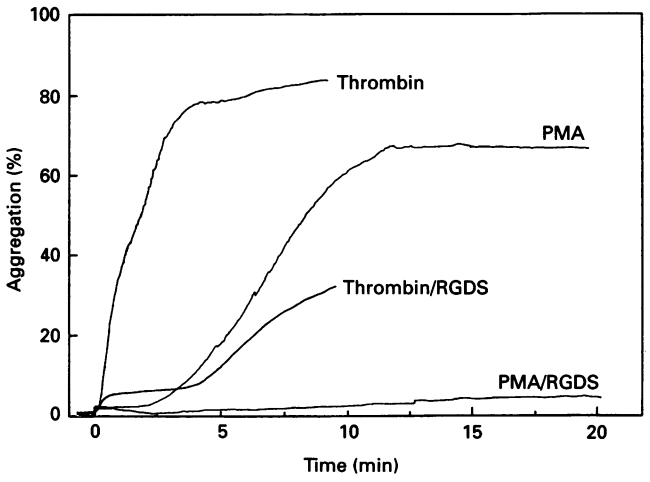

Figure 3 Effects of RGDS on platelet aggregation

Platelets were treated with 500 μM RGDS (or control buffer) for 20 min at 37 °C in a thermomixer, and then challenged with 0.3 unit/ml thrombin or 300 nM PMA for 10 or 20 min respectively in an aggregometer, as described in the Materials and methods section. Aggregation is expressed as percentage light transmittance, which increases as platelets form aggregates, relative to buffer alone (= 100%).

are due to the activation of protein kinase C. Ro-31-8220 was found to inhibit the activation of PLD induced by A23187 (Figure 2) over a similar concentration range to that causing inhibition of thrombin-stimulated activity (Figure 1). It has been demonstrated that 3–10 μM Ro-31-8220 inhibits A23187-mediated pleckstrin phosphorylation [31], indicating that protein kinase C activity is indeed inhibited under these conditions. When a maximally effective concentration of A23187 was added to platelets together with PMA, the resultant PLD activity was less than the sum of the effects of these agents added alone (results not shown), which would occur if these two agonists activated PLD via a common pathway. These data suggest that Ca^{2+} activates PLD via a protein kinase C-dependent mechanism.

Based on these results and experiments showing that Ro-31-8220 inhibits platelet aggregation ([31]; A. Greinacher, unpublished work), it seemed possible that activation of PLD might depend on the aggregation of platelets (i.e. it might occur downstream of fibrinogen binding and aggregation). To test this hypothesis, we used a peptide antagonist of integrin $\alpha_{IIb}\beta_3$ having the sequence arginine-glycine-aspartate-serine (RGDS) that interferes with the binding of fibrinogen [32]. In the presence of 500 μM RGDS, platelet aggregation in response to 300 nM PMA was totally inhibited, and aggregation induced by 0.3 unit/ml thrombin was delayed and significantly reduced (Figure 3). Under these conditions, however, activation of PLD by thrombin and PMA was not blocked by the presence of RGDS (Table 2). In a control experiment, RGES, an analogous peptide that does not bind to integrin $\alpha_{IIb}\beta_3$, did not affect aggregation or PLD activity (results not shown). Taken together, these data indicate that PLD is activated in platelets even when aggregation is inhibited.

Integrin $\alpha_{IIb}\beta_3$ must first be activated by an intracellular signal in order to bind fibrinogen and initiate platelet aggregation [20]. To answer the question of whether activation of integrin $\alpha_{IIb}\beta_3$ is required for the control of PLD, we assayed PLD activity in platelets from a patient with type I Glanzmann's thrombasthenia that lack the integrin $\alpha_{IIb}\beta_3$ complex [33] and therefore do not aggregate in response to thrombin or PMA. We determined that

Table 2 Effects of RGDS on thrombin- and PMA-stimulated PLD activity

[^{3}H]Myristic-acid-labelled platelets were treated with the indicated concentrations of RGDS or buffer alone as in the legend to Figure 3, and subsequently with 0.3 unit/ml thrombin or 300 nM PMA in the presence of 0.5% ethanol. PLD activity was measured as described in the Materials and methods section and is expressed relative to the thrombin response as in Table 1. Data represent means (±S.E.M.) of determinations from the numbers of experiments shown in parentheses. Differences between data for RGDS-pretreated and untreated samples were not significant ($P > 0.05$).

		PLD activity (%)		
Treatment	Pretreatment...	None	500 μM RGDS	1 mM RGDS
Thrombin		100	120±15 (8)	111±17 (10)
PMA		56±7 (14)	45±8 (13)	58±9 (7)

Table 3 Activation of PLD in platelets from a patient with Glanzmann's thrombasthenia

Platelets were prepared and labelled with [^{3}H]myristic acid as described in the Materials and methods section. Suspensions were then treated in the presence of 0.5% ethanol with 0.3 unit/ml thrombin for 10 min or with 300 nM PMA for 20 min, after which [^{3}H]PEt was quantified. Levels of [^{3}H]PEt (with background radioactivity subtracted) are shown as means (±S.E.M.) or range, with the numbers of replicates shown in parentheses. All values were significantly different from zero ($P < 0.05$).

	[^{3}H]PEt (d.p.m./sample)	
Treatment	Expt. 1	Expt. 2
Thrombin	948±92 (2)	749±12 (3)
PMA	98±28 (3)	179±10 (3)

thrombin and PMA were able to activate PLD in platelets from this patient (Table 3), demonstrating that the activation of PLD still occurs when the integrin $\alpha_{IIb}\beta_3$ complex is absent. Protein kinase C is able to be activated by thrombin in this patient's platelets, as determined by the phosphorylation of pleckstrin (U. Liebenhoff, personal communication).

DISCUSSION

The widespread occurrence of PLD and its ability to be rapidly activated by a variety of cellular stimuli point to a role for this enzyme in cellular signalling. The mechanisms by which PLD is activated appear to vary between cell types and stimulatory signals. Our data show that protein kinase C regulates, directly or indirectly, the activity of PLD in human platelets. This conclusion derives from our findings that (i) agonists such as thrombin, collagen and others that lead to activation of protein kinase C in platelets also activate PLD, (ii) PMA, a direct activator of protein kinase C, also activates PLD, and (iii) Ro-31-8220 inhibits thrombin- and PMA-activated PLD under conditions in which it also inhibits protein kinase C.

Our results implicating protein kinase C in the activation mechanism of PLD are supported by reports that a synthetic diacylglycerol caused the release of choline from platelets [10], and that pretreatment of platelets with PMA enhanced the production of PEt measured subsequently in platelet membranes [17]. Huang et al. [9], however, found only a very slight increase in [^{3}H]PA formation in platelets in response to phorbol dibutyrate, and 10 μM staurosporine inhibited the increase produced by thrombin by only 18%. These authors treated platelets with phorbol ester for only 5 min, whereas we observed no significant increase in [^{3}H]PEt until time points after 5 min of PMA treatment (S. Scheible and E. A. Martinson, unpublished work), consistent with the delayed onset of aggregation in response to this agonist compared with that induced by thrombin (Figure 3). In our hands, staurosporine inhibited thrombin-stimulated PLD activity by more than 80% at 10 μM (S. Scheible, unpublished work), both at 0.3 unit/ml thrombin and at the supramaximal concentration of 5 units/ml used by Huang et al. [9]. The conclusion of these authors that PLD is primarily regulated by Ca^{2+} [9], based on the stimulatory effect of A23187, does not take into account the ability of A23187 to activate protein kinase C in platelets [31]. This is likely to be the mechanism by which this ionophore activates PLD, since Ro-31-8220 inhibits A23187-stimulated PLD activity (Figure 2). Our results, however, do not exclude the possibility that intracellular Ca^{2+} plays a role in the activation of PLD, via protein kinase C, during physiological platelet activation. Alternatively, the differences between the results of Huang et al. [9] and ours might be further explained by their use of a different radioactive label ([^{3}H]lysophosphatidylcholine containing a 1-*O*-alkyl linkage) or their measurement of the cellular metabolite [^{3}H]PA instead of the PLD-specific product [^{3}H]PEt. It could be that their method measures the activity of a different isoform of PLD, since a different radiolabelled substrate (1-*O*-alkyl- versus 1-*O*-acyl-phosphatidylcholine) would be utilized. Based on these discrepant results, we can speculate that there may be at least two PLD isoforms in platelets, one regulated by protein kinase C and one activated by a protein kinase C-independent pathway. A similar conclusion was reached by Song and Foster [25], who examined PLD activation in fibroblasts using different radioactive precursors to label phospholipids. The gaining of more precise information about these regulatory mechanisms awaits the purification, sequencing and cloning of PLD.

The PLD of platelets that we have investigated, then, appears to belong to the group of PLD activities that are regulated by protein kinase C in various cell types [2]. Questions remaining are whether platelet PLD activation, in turn, results in diacylglycerol formation leading to a later, sustained, increase in protein kinase C activity, and what role this might play in platelet physiology. Along these lines, activation of protein kinase C has been shown to be involved in secondary platelet aggregation and secretion, which occurs 0.5–2 min following stimulation [34]. Alternatively, it could be other second messengers such as PA or lysoPA that are important in a PLD-initiated signalling cascade. It has recently been demonstrated that platelets contain a novel, PA-activated, protein kinase [35].

Although activation of PLD may be measured by the production of choline or PA, we chose to measure the formation of the PLD-specific product PEt because it is possible that the aforementioned products could arise from other cellular metabolic pathways. It is known that ethanol has inhibitory effects on platelet aggregation and secretion in response to low (< 0.1 unit/ml) concentrations of thrombin [36,37]. Our use of 0.5% (v/v; 86 mM) ethanol during platelet incubations, however, which was required to measure the formation of PEt, had no significant effect on aggregation in response to 0.3 unit/ml thrombin (S. Scheible and E. A. Martinson, unpublished work). In addition, it has been demonstrated that 150 mM ethanol has no effect on inositol phospholipid hydrolysis, Ca^{2+} mobilization or pleckstrin phosphorylation in platelets exposed to thrombin [37].

We have demonstrated previously that a number of inhibitors of protein tyrosine kinases inhibit thrombin-stimulated PLD activity in human platelets [11]. Conversely, peroxyvanadate,

which is known to inhibit protein tyrosine phosphatases and cause platelet aggregation, activates PLD ([11], and references therein). These observations point to a possible role for protein tyrosine kinases in the regulation of PLD in platelets, a role that has been investigated in other cell types [2,25,38–40]. The relationship between protein tyrosine kinases and protein kinase C in the activation of PLD is unknown; one report has shown that the PLD activity stimulated in fibroblasts transformed by the protein tyrosine kinase v-Src is protein kinase C-independent and distinct from that stimulated by phorbol esters [25]. Results from our laboratory, however, are consistent with the possibility that phosphorylation of the major protein tyrosine kinase in platelets, pp60$^{c\text{-}src}$, by protein kinase C during platelet activation might be important for the translocation and activation of this enzyme [41,42]. This could mean that protein tyrosine kinases such as pp60$^{c\text{-}src}$ activate PLD downstream of protein kinase C in the signalling cascade. We have also recently demonstrated that protein tyrosine kinase inhibitors decrease guanine nucleotide-stimulated PLD activity in permeabilized platelets [11]. Thus the role of G-proteins in the activation of PLD in platelets, suggested by the ability of GTP analogues to activate PLD in permeabilized platelets [11,43] and platelet membranes [17], also remains to be clarified. Two laboratories have recently reported that a small GTP-binding protein, ADP-ribosylation factor (denoted ARF), mediates the effects of guanine nucleotide stimulation of PLD from HL60 cells *in vitro* [44,45].

Since agonists that activate PLD (Table 1) also cause aggregation, and inhibition of PLD by Ro-31-8220 (Figures 1 and 2) occurs under conditions where aggregation is also inhibited ([31]; A. Greinacher, unpublished work), we examined whether aggregation of platelets is necessary for the activation of PLD to occur. Under conditions in which aggregation was absent or severely impaired in the presence of RGDS, PLD was still able to be activated in response to thrombin or PMA (Table 2). These results show that aggregation does not mediate PLD activation, and corroborate data showing that choline release in response to thrombin occurs in unstirred platelet suspensions [10].

The platelet receptor for fibrinogen, integrin $\alpha_{IIb}\beta_3$, participates in some of the intracellular responses that occur during platelet aggregation. Integrin $\alpha_{IIb}\beta_3$ was recently found to mediate activation of platelet PLD by high-density lipoproteins, which are thought to interact with this glycoprotein complex [46]. In contrast, in platelets from a Glanzmann's patient that lack the integrin $\alpha_{IIb}\beta_3$ complex, we found that thrombin and PMA activated PLD (Table 3), demonstrating that this glycoprotein does not play a role in PLD activation by thrombin or PMA.

The fact that thrombin activation of PLD does not depend on prior activation of integrin $\alpha_{IIb}\beta_3$ is consistent with the possibility that the converse is true: namely, that PLD might be involved in the 'inside-out' signalling known to be responsible for the induction of the integrin complex that is necessary for fibrinogen binding and aggregation [20]. A number of events during platelet activation are dependent on the binding of fibrinogen, including translocation of pp60$^{c\text{-}src}$ from the plasma membrane to a cytoskeletal fraction [47], and phosphorylation [48] and dephosphorylation [49,50] of certain proteins on tyrosine residues. Other events, however, such as the activation of pp60$^{c\text{-}src}$ [51], tyrosine phosphorylation of other proteins [52] and protein kinase C activation (U. Liebenhoff, personal communication), are integrin $\alpha_{IIb}\beta_3$-independent. Responses such as PLD activation that fall into this latter category may be involved in the induction of integrin $\alpha_{IIb}\beta_3$ and platelet aggregation. Our results do not rule out the possibility that PLD is activated in parallel with these latter events and does not participate in aggregation, but the finding that PA, the direct product of PLD, and its metabolite lysoPA enhance the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ *in vitro* [14] lend support to the hypothesis that PLD is important to integrin activation and platelet function.

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