Phosphatidylinositol 4,5-bisphosphate is selectively retained by platelet–fibrin clots formed by thrombin

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Stimulation of human or rabbit platelets with thrombin in the presence of fibrinogen caused a large decrease, compared with unstimulated controls, in the amount of phosphatidylinositol 4,5-bisphosphate (PIP₂) that could be extracted with acidified chloroform/methanol (60% at 60 s). In contrast, stimulation in the absence of added fibrinogen increased the amount of PIP₂. The decrease was specific for PIP₂, because similar decreases could not be demonstrated for other phosphoinositides or phospholipids. The interaction of polymerizing fibrin with stimulated platelets was required for the decrease in PIP₂, since polymerized fibrin formed by reptilase did not cause the decrease in the amount of extractable PIP₂, and inhibition by glycyl-L-prolyl-L-arginyl-L-proline of polymerization of fibrin formed by the action of thrombin prevented the large decrease in extractable PIP₂. The decrease in extractable PIP₂ could not be explained by increased degradation of PIP₂, since sufficient degradation products were not formed. Thus, when platelets are stimulated with thrombin in the presence of fibrinogen, an association of polymerizing fibrin with the stimulated platelets occurs that leads to decreased extractability of PIP₂. This may mean that PIP₂ forms a specific association with platelet proteins that are involved in clot retraction.

INTRODUCTION

Membrane lipids in cells can associate with cellular proteins, including the cytoskeleton (Anderson & Marchesi, 1985; Burn *et al.*, 1985; Ferguson *et al.*, 1985; Futerman *et al.*, 1985; Lassing & Lindberg, 1985; Low & Finean, 1977, 1978; Meyer *et al.*, 1982; Schick *et al.*, 1983). Low *et al.* (1986) have proposed that the enzymes which are released from the surface of various types of cells by PI-specific phospholipase C may be covalently attached to PI, which serves as their membrane attachment site. For platelets, there is evidence of association of membrane lipids with protein when the platelets are stimulated with thrombin (Burn *et al.*, 1985; Schick *et al.*, 1983). Lassing & Lindberg (1985) have described a specific interaction between PIP₂ and profilactin which promotes the dissociation of the profilactin complex, leaving a profilin-PIP₂ complex and freeing actin, which then polymerizes.

In studies of human and rabbit platelets stimulated with thrombin in the presence of fibrinogen, we have found an unexpectedly large decrease in the amount of PIP₂ that can be recovered by extraction with acidified chloroform/methanol. Thrombin stimulates platelets to change shape, aggregate and secrete the contents of their storage granules, as well as converting fibrinogen into fibrin. Retraction of the platelet–fibrin clot follows, the mechanical force being provided by the contractile system of the platelets. The interaction of platelets with polymerizing fibrin is a key step in clot retraction and consolidation of haemostatic plugs.

To examine the reason for the decreased recovery of PIP₂ from platelets stimulated with thrombin in the presence of fibrinogen, we have examined whether the decrease is due to (1) inaccessibility to extracting solvents

of platelet membranes in the centre of the clot (Zucker & Masiello, 1983), by comparing PIP₂ extraction with that of other phospholipids, (2) increased degradation of PIP₂ by phospholipase C, by measuring formation of IP₃, (3) non-specific association of PIP₂ with protein during extraction, which prevents its extraction, or (4) a specific association of PIP₂ with platelet protein as a result of platelet stimulation and interaction with polymerized fibrin, which prevents its extraction.

METHODS

Platelet preparation

Human platelets from whole blood, taken from the forearm vein into ACD (Aster & Jandl, 1964), were washed once in Tyrode solution containing heparin (50 units/ml), albumin (0.35%), glucose (0.1%) and apyrase (Mustard *et al.*, 1972). After centrifugation, the platelets were resuspended in a modified Tyrode solution containing albumin, glucose and apyrase, from which phosphate was omitted; buffering was provided by the addition of 5 mM-Hepes. Rabbit platelets were collected and resuspended in a Ca²⁺- and phosphate-free Tyrode solution (containing albumin, glucose, apyrase and 5 mM-Hepes) as previously described (Vickers *et al.*, 1982).

Studies of changes in amounts of phospholipids in thrombin-stimulated human or rabbit platelets

Suspensions of either human or rabbit platelets were incubated for 1 h with [³²P]PI₁ (20 µCi/ml; New England Nuclear, Dorval, PQ, Canada) which was used to locate the phospholipids on the thin-layer chromatograms in studies of changes in the amounts of the phospholipids.

Abbreviations used: PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; IP₃, inositol trisphosphate; IP₂, inositol bisphosphate; IP, inositol phosphate; GPRP, glycyl-L-prolyl-L-arginyl-L-proline.

The human platelets were freed of unincorporated label by centrifugation and resuspension in Tyrode solution (containing phosphate, albumin, glucose and apyrase). Rabbit platelets were freed of unincorporated label by centrifugation and resuspension in Ca^{2+} -free Tyrode solution containing phosphate. In both cases, the platelets were finally resuspended in Tyrode solution [containing albumin (0.35%), glucose (0.1%) and apyrase] at 10^9 platelets/ml. The platelets were stimulated with thrombin while being stirred in a cuvette in an aggregometer (Payton Associates, Scarborough, Ont., Canada), and 1 ml of reaction mixture was extracted with 3.75 ml of chloroform/methanol (1:2, v/v), followed by extraction with acidified chloroform/methanol as previously described (Vickers *et al.*, 1982). The chloroform phases were combined and the phospholipids were fractionated on silica-gel H t.l.c. plates (Canlab, Toronto, Ont., Canada) in two dimensions (Vickers *et al.*, 1982). The lipids were localized by viewing the plates under u.v. light after spraying with Rhodamine 6G and by autoradiography. The spots containing the phospholipids were scraped from the plates, and the phospholipids were digested with HClO_4 . After addition of water, and centrifugation (1000 *g* for 10 min), samples of the supernatant were removed for determination of P_i and incorporated label, as previously described (Vickers *et al.*, 1982). To quantify the label that was not in the chloroform phases, the aqueous/methanol phase, including the protein interface, was centrifuged (250 *g* for 10 min) after removal of the chloroform phase. Portions of the aqueous/methanol phase (200 μl) were removed and dried in scintillation vials for counting after addition of ACS (Amersham/Searle, Mississauga, Ont., Canada) cocktail. The remaining aqueous/methanol phase was then removed and the protein solubilized with NCS (Amersham/Searle), transferred to scintillation vials and counted for radioactivity in ACS cocktail.

Studies of changes in inositol labelling of phospholipids and inositol phosphates in thrombin- or ADP-stimulated rabbit platelets

For determination of labelling of the phosphoinositides and inositol phosphates, the platelets were prelabelled as described in the previous section, except that [^3H]inositol (20 $\mu\text{Ci}/\text{ml}$; American Radiolabeled Chemicals, St. Louis, MO, U.S.A.) was included with the [^{32}P]PI $_i$. Phosphoinositide extraction and fractionation were done as described above.

For inositol phosphate studies, the platelets were incubated with 20 mM-Li $^+$ for 1 h after labelling; Li $^+$ blocks degradation of the inositol phosphates to inositol and phosphate. Inositol phosphates were extracted with 10% trichloroacetic acid and fractionated on ion-exchange columns [AG1-X8; Bio-Rad Laboratories (Canada), Mississauga, Ont., Canada] as previously described (Vickers *et al.*, 1984).

The data are presented as means $\pm$ S.E.M. and the significance of the data was assessed by paired *t*-test analysis.

RESULTS

Stimulation of human platelets with thrombin for 10 s in the presence of fibrinogen decreased the amount of extractable PIP $_2$ by 32% ($P < 0.01$, $n = 6$), compared

with an 11% decrease ($P < 0.01$, $n = 6$) in the absence of fibrinogen. As shown in Table 1, the decrease was even greater by 60 s. In platelets stimulated with thrombin in the presence of fibrinogen, PIP $_2$ decreased by 60%, whereas in the absence of fibrinogen the initial decrease in amount of extractable PIP $_2$ was replaced by an increase in amount to approx. 123% of the control value. To account for the disappearance of PIP $_2$, the label which remained in the aqueous/methanol phase and the protein interface was determined in experiments with platelets prelabelled with [^3H]inositol. The isolated protein precipitate from platelets stimulated with thrombin in the absence of fibrinogen contained 6942 ± 1236 d.p.m./ 10^9 platelets. In contrast, the radioactivity in the protein precipitate was increased by approx. 4300 d.p.m. in platelets stimulated in the presence of fibrinogen (11216 ± 1628 d.p.m./ 10^9 platelets; $n = 2$). There was no increase in the amount of label in the aqueous/methanol phase in the presence of fibrinogen compared with that in its absence. The extraction of PIP $_2$ was then compared with that of the other phospholipids from platelets stimulated with thrombin in the absence and in the presence of fibrinogen. In the absence or the presence of fibrinogen, stimulation of the platelets with thrombin did not significantly change the amount of extractable PIP (Table 1). The decrease in amount of PI and the increases in amount of PA in thrombin-stimulated platelets were similar in the absence or the presence of fibrinogen (Table 1). The amount of extractable PC was not significantly changed by stimulation of the platelets with thrombin in either the absence or the presence of fibrinogen (Table 1).

Thrombin stimulation of rabbit platelets in the presence of fibrinogen had an effect, similar to the effect of stimulation of human platelets, on the amounts of PIP $_2$ that were extracted (Table 2). As indicated in Table 3, the amount of label recovered in PIP $_2$ in platelets stimulated in the presence of fibrinogen was also decreased compared with platelets stimulated in the absence of fibrinogen (by 5218 d.p.m. of [^3H]inositol/ 10^9 platelets). The label remaining in the aqueous/methanol phase and the protein interface was also determined in experiments with rabbit platelets prelabelled with [^3H]inositol. The isolated protein precipitate from platelets stimulated with thrombin in the absence of fibrinogen contained 4153 ± 1355 d.p.m./ 10^9 platelets. In contrast, the radioactivity in the protein precipitate was increased by approx. 5200 d.p.m. in platelets stimulated in the presence of fibrinogen (9345 ± 853 d.p.m./ 10^9 platelets, $n = 6$). There was no increase in the amount of label in the aqueous/methanol phase in the presence of fibrinogen compared with that in its absence. Since the other phospholipids examined were recovered in the same amounts in the presence or the absence of fibrinogen with both human and rabbit platelets, the failure to extract PIP $_2$ after stimulation of platelets with thrombin in the presence of fibrinogen is unlikely to be due to failure to remove phospholipid from the core of the platelet-fibrin clot.

In contrast with thrombin-stimulated platelets, the presence of fibrinogen had no significant effect on the recovery of PIP $_2$ from ADP-stimulated platelets. Stimulation of rabbit platelets with 10 μM -ADP in the absence of added fibrinogen resulted in a decrease in the amount of PIP $_2$, by 0.33 nmol/ 10^9 platelets. ADP stimulation in

Table 1. Effect of stimulating washed human platelets with 1 unit of thrombin/ml of suspension in the absence and presence of fibrinogen (0.4 mg/ml) for 60 s on the amounts of phospholipids extracted from the incubation mixture

Human platelets were washed, prelabelled with [32 P]P_i, and the effect of thrombin stimulation on amounts of the phospholipids was determined as described in the Methods section. Significance of the data, which are presented as means $\pm$ S.E.M., was assessed by paired *t*-test analysis: ns. not significant.

	Amount of phospholipid (nmol/10 ⁹ platelets)			
	No fibrinogen (<i>n</i> = 8)		+ Fibrinogen (<i>n</i> = 6)	
	Tyrode solution	+ Thrombin	Tyrode solution	+ Thrombin
PIP ₂	3.83 $\pm$ 0.14	4.7 $\pm$ 0.24	3.84 $\pm$ 0.44	1.62 $\pm$ 0.39
PIP	3.70 $\pm$ 0.28	4.37 $\pm$ 0.31	3.53 $\pm$ 0.40	3.20 $\pm$ 0.61
PI	24.2 $\pm$ 1.1	18.1 $\pm$ 1.7	23.1 $\pm$ 2.6	18.5 $\pm$ 1.9
PA	1.39 $\pm$ 0.17	4.07 $\pm$ 0.41	1.72 $\pm$ 0.21	4.35 $\pm$ 0.66
PC	203 $\pm$ 8.3	197 $\pm$ 8.7	198 $\pm$ 23	179 $\pm$ 20
		ns		ns

Table 2. Effect of stimulating washed rabbit platelets with 1 unit of thrombin/ml of suspension in the absence and presence of fibrinogen (0.4 mg/ml) for 60 s on the amounts of phospholipids extracted from the incubation mixture

Rabbit platelets were washed, prelabelled with [32 P]P_i, and the effect of thrombin stimulation on the phospholipid was analysed as described in the Methods section. Significance of the data, which are presented as means $\pm$ S.E.M. (*n* = 8), was assessed by paired *t*-test analysis: ns, not significant.

	Amount of phospholipid (nmol/10 ⁹ platelets)			
	No fibrinogen		+ Fibrinogen	
	Tyrode solution	+ Thrombin	Tyrode solution	+ Thrombin
PIP ₂	3.23 $\pm$ 0.22	3.41 $\pm$ 0.25	3.04 $\pm$ 0.21	1.33 $\pm$ 0.29
PIP	2.66 $\pm$ 0.28	3.28 $\pm$ 0.26	2.42 $\pm$ 0.25	3.41 $\pm$ 0.26
PI	16.0 $\pm$ 0.65	12.2 $\pm$ 1.07	15.7 $\pm$ 0.81	11.8 $\pm$ 0.95
PA	2.14 $\pm$ 0.44	3.79 $\pm$ 0.45	1.73 $\pm$ 0.53	3.11 $\pm$ 0.75
PC	102 $\pm$ 4.3	103 $\pm$ 4.8	95.3 $\pm$ 5.3	96.4 $\pm$ 3.2
		ns		ns

the presence of fibrinogen resulted in a decrease in the amount of PIP₂, by 0.31 nmol/10⁹ platelets (*n* = 4).

If the formation of a platelet-fibrin clot is required for the retention of PIP₂, blocking polymerization of fibrin might be expected to eliminate the effect. The tetrapeptide GPRP prevents the polymerization of fibrin monomers (Laudano & Doolittle, 1978) and thereby prevents formation of a platelet-fibrin clot. GPRP (0.4 mM) does not abolish binding of fibrin monomers to platelets or platelet aggregation (Harfenist *et al.*, 1985). In the present experiments, GPRP prevented the large decrease in amount of extractable PIP₂ when human platelets were stimulated with thrombin for 60 s in the presence of fibrinogen (the amount of PIP₂ recovered from control samples with fibrinogen and GPRP was 3.68 $\pm$ 0.07 nmol/10⁹ platelets; from thrombin-stimu-

lated platelets in the presence of fibrinogen and GPRP, 4.37 $\pm$ 0.30 nmol/10⁹ platelets; *n* = 4). These findings in the presence of GPRP are similar to those obtained with platelets stimulated with thrombin in the absence of added fibrinogen (Table 1). GPRP alone had no effect on the amount of PIP₂ recovered from platelets stimulated with thrombin in the absence of fibrinogen (*n* = 4). Thus the association of stimulated platelets with polymerizing fibrin in a platelet-fibrin clot appears to be required for the retention of PIP₂.

Another explanation for the failure to extract PIP₂ could be that it associates with denatured proteins during extraction and thus is not extracted. Since reptilase causes the polymerization of fibrin, but does not stimulate platelets (Niewiarowski *et al.*, 1972), we measured the amount of PIP₂ that could be extracted

Table 3. Effect of stimulating washed rabbit platelets with 1 unit of thrombin/ml of suspension for 60 s in the absence and presence of fibrinogen (0.4 mg/ml) on the amount of label in the extractable phosphoinositides and their derivatives

The results are presented as means $\pm$ S.E.M. ($n = 4$), with the differences (Δ) in labelling between samples incubated with Tyrode solution and samples treated with thrombin. The differences between the thrombin-induced changes in PIP_2 and IP_3 in the absence and presence of fibrinogen were the only such differences that were statistically significant. Platelet preparation, labelling and determination of labelling of the phosphoinositides and inositol phosphates are described in the Methods section. The data for the inositol phosphates and the data for the phospholipids were obtained in separate experiments.

	[^3H]Inositol labelling and changes due to thrombin (d.p.m./ 10^9 platelets)					
	No fibrinogen			+ Fibrinogen		
	Tyrode solution	Thrombin		Tyrode solution	Thrombin	
PIP_2	6463 ± 1291	6853 ± 1136		7541 ± 188	1635 ± 188	
PIP	2711 ± 357	4103 ± 517	$\Delta = +390$	2190 ± 612	3586 ± 1178	$\Delta = -5906$
PI	40104 ± 5598	24125 ± 2820	$\Delta = +1392$	42077 ± 6042	27874 ± 1686	$\Delta = +1396$
Inositol	101802 ± 17428	99018 ± 16907	$\Delta = -15979$	96952 ± 16701	94600 ± 16747	$\Delta = -14203$
Glycero-3-phosphoinositol	2900 ± 665	5614 ± 1510	$\Delta = -2784$	4101 ± 1325	7698 ± 3181	$\Delta = -2352$
IP	2511 ± 315	8398 ± 198	$\Delta = +2714$	2995 ± 124	9378 ± 648	$\Delta = +3597$
IP_2	1402 ± 35	10128 ± 2650	$\Delta = +5887$	2187 ± 480	9375 ± 2197	$\Delta = +6383$
IP_3	402 ± 99	658 ± 166	$\Delta = +8726$	608 ± 229	677 ± 197	$\Delta = +7188$
			$\Delta = +256$			$\Delta = +69$
Sum of Δ values . . .		+ 602			- 3828	

from rabbit platelets in the presence of fibrinogen and the amount that could be extracted in the presence of polymerizing fibrin produced by the action of reptilase on fibrinogen. Under these experimental conditions, 3.18 ± 0.07 nmol of $\text{PIP}_2/10^9$ platelets was recovered by extraction in the presence of fibrinogen and 3.06 ± 0.05 nmol of $\text{PIP}_2/10^9$ platelets was recovered in the presence of polymerizing fibrin. Thus, neither the presence of fibrinogen nor the formation of polymerizing fibrin, in circumstances in which platelets are not stimulated, leads to a decrease in extractable PIP_2 .

To assess whether or not the decrease in recovery of PIP_2 could be due to increased degradation of PIP_2 to IP_3 , the changes in [^3H]inositol labelling of the phosphoinositides and other inositol-containing species recovered from the platelets were determined in platelets stimulated with thrombin in the absence and presence of fibrinogen. The decrease in extractable PIP_2 in platelets stimulated with thrombin in the presence of fibrinogen could not be accounted for by increased formation of the inositol phosphates (Table 3). These data, and the observation that thrombin stimulation of platelets in the presence of fibrinogen did not cause a larger increase in PA than did stimulation in the absence of fibrinogen (Tables 1 and 2), indicate that degradation of PIP_2 by phospholipase C had not been increased. The absence of an increase in PIP large enough to account for the decrease in PIP_2 (Tables 1 and 2) indicates that increased degradation of PIP_2 by phosphomonoesterase or decreased synthesis of PIP_2 from PIP cannot account for the large decrease in PIP_2 . The sum of the changes in

labelling of the inositol-containing species in the platelets stimulated in the presence of fibrinogen is about 4400 d.p.m./ 10^9 platelets less than in platelets stimulated in the absence of fibrinogen. Thus these results are consistent with the hypothesis that the decrease is mainly due to failure to extract PIP_2 . Although the lysophosphoinositides were not quantified and thus not included in this accounting, there was no evidence from the autoradiograms that the labelling of these species was changed by the presence of fibrinogen during thrombin stimulation of the platelets.

DISCUSSION

The results of these experiments demonstrate that stimulation of human or rabbit platelets with thrombin in the presence of fibrinogen results in the formation of a platelet-fibrin clot and in a decrease in the amount of PIP_2 that can be extracted that is much larger than the decrease caused by degradation of PIP_2 to IP_3 by phospholipase C. This is specific for PIP_2 , since the other phospholipids examined can be recovered in the same amounts in the presence of fibrinogen as in its absence. The thrombin-stimulated changes in extractable PIP_2 found in the absence of fibrinogen are similar to changes reported by others (Billah & Lapetina, 1982; Broekman, 1984; Holmsen *et al.*, 1984; Perret *et al.*, 1983; Rendu *et al.*, 1983; Wilson *et al.*, 1985). The observations that PIP_2 can be extracted from unstimulated platelets in the presence of fibrinogen, or from unstimulated platelets

exposed to polymerizing fibrin formed by reptilase, and that PIP_2 can be extracted from thrombin-stimulated platelets when polymerization of fibrin is blocked by GPRP, indicate that the failure to extract PIP_2 is unlikely to be due to non-specific association of the PIP_2 with protein in the extraction mixture. These observations, and the observation that ADP stimulation of rabbit platelets in the presence of fibrinogen did not decrease recovery of PIP_2 , although ADP-stimulated platelets are known to bind fibrinogen (Mustard *et al.*, 1978), are consistent with the retention of PIP_2 in thrombin-stimulated platelets requiring interaction of polymerizing fibrin with stimulated platelets. There is no evidence of increased PIP_2 degradation by one of its usual metabolic pathways, since there was no increase in the amounts of PIP , PI , PA , IP_3 , IP_2 , IP or the lysophosphoinositides to account for the decrease in PIP_2 . A possible explanation of the results is that the PIP_2 specifically associates with platelet proteins, so that very little can be extracted. Whether or not this association is similar to the PIP_2 -profilactin association reported by Lassing & Lindberg (1985) is not known. Since GPRP prevented retention of PIP_2 in human platelets stimulated with thrombin in the presence of fibrinogen, and since monomeric fibrin binds to platelets in the presence of GPRP at the concentration used in the present experiments (Harfenist *et al.*, 1985), this may mean that polymerizing fibrin has an additional form of association with the platelet membrane compared with monomeric fibrin and that this involves PIP_2 and platelet proteins [possibly cytoskeletal proteins, since there are major changes in the associations among these proteins when platelets are stimulated with thrombin (Fox, 1985)].

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