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Aggregation-dependent, integrin-mediated increases in cytoskeletally associated PtdInsP$_2$ (4,5) levels in human platelets are controlled by translocation of PtdIns 4-P 5-kinase C to the cytoskeleton

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Thrombin-stimulated aggregation of human platelets promotes an increase in the phosphatidylinositol 4-phosphate (PtdIns 4-P) 5-kinase (PIPkin) activity in the cytoskeleton. This phenomenon is associated with translocation of PIPkin isoform C to the cytoskeleton and with an increase in the amount of phosphatidylinositol bisphosphate (PtdInsP$_2$) bound to the cytoskeletal pellet. All three of these effects are prevented if the platelets are not stirred or if RGD-containing peptides are present, demonstrating that they require integrin activation. All three are also abolished by pretreatment with okadaic acid, which also prevents the aggregation-dependent translocation of pp60$^{c\text{-}src}$ to the cytoskeleton. The results point to the existence of a cytoskeletally associated PtdInsP$_2$ pool under the control of integrin-mediated signals that act via PIPkin C and suggest that a common, okadaic acid-sensitive mechanism may underlie the aggregation-dependent translocation of certain signalling molecules to the platelet cytoskeleton.

Keywords: cytoskeleton/integrin/PtdIns 4,5-P$_2$/platelet

Introduction

The phospholipid phosphatidylinositol bisphosphate (PtdInsP$_2$) is best known as the precursor of the second messengers inositol 1,4,5-triphosphate (Ins 1,4,5-P$_3$) and diacylglycerol, which mobilize intracellular Ca^{2+} stores and activate various protein kinase C isoforms respectively (Berridge and Irvine, 1989). It has recently become clear, however, that PtdInsP$_2$ is also intimately involved in a variety of other cellular processes and fulfils several distinct intracellular functions. First, it provides the physiological substrate for the phosphoinositide 3-kinase (PI 3-kinase) pathway, which generates the putative second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P$_3$) (Stephens *et al.*, 1991). PtdInsP$_2$ also modulates the activities of various actin binding proteins *in vitro*, leading to the suggestion that it may regulate cytoskeletal structure and function (Janmey, 1994). Additionally, PtdInsP$_2$ has been implicated in regulating vesicle secretion (Hay *et al.*, 1995), possibly via inhibition of a protein kinase (Gross *et al.*, 1995). PtdInsP$_2$ may also regulate the small GTP binding protein ARF (Randazzo and Kahn, 1994), implying a possible role in vesicle trafficking, and acts as a co-factor in the regulation of a subtype of phospholipase D (Liscovitch *et al.*, 1994). It may also regulate the localization or function of PH domain-containing proteins (Harlan *et al.*, 1994) and has been reported to increase the activity of the protein kinase C-related kinase PRK-1 (Palmer *et al.*, 1995). Despite the central importance of PtdInsP$_2$ to cellular function, however, much remains to be discovered about the enzymes that produce it, the phosphatidylinositol 4-bisphosphate (PtdIns 4-P) 5-kinases (PIPkins).

Several PIPkin isozymes have now been isolated from a variety of tissues (Van Dongen *et al.*, 1984; Ling *et al.*, 1989; Bazenet *et al.*, 1990; Moritz *et al.*, 1990; Divecha *et al.*, 1992). We have previously identified three different PIPkin activities in bovine brain and purified one of them, PIPkin C, to homogeneity (Divecha *et al.*, 1992). This isoform is identical to one purified from human erythrocytes which has been termed the type II isozyme (Bazenet *et al.*, 1990; Brooksbank *et al.*, 1993). It has recently been cloned (Boronenkov and Anderson, 1995; Divecha *et al.*, 1995) and lacks homology with previously cloned PtdIns 3- and 4-kinases, suggesting that the PIPkins may represent a novel family of lipid kinase enzymes.

In addition to PIPkin C/type II, an immunologically distinct 68 kDa PIPkin, type I, has also been identified (Bazenet *et al.*, 1990). Antibodies against type I recognize an additional 90 kDa PIPkin (Jenkins *et al.*, 1994); these immunologically related isoforms are now referred to as types Ia and Ib respectively. Type I PIPkins can reconstitute ATP-dependent vesicle secretion in permeabilized PC12 cells, but type II/PIPkin C cannot (Hay *et al.*, 1995), suggesting that distinct isoforms may regulate different functions of PtdInsP$_2$. Such functional compartmentalization may underlie the involvement of PtdInsP$_2$ in so many distinct cellular processes (Liscovitch and Cantley, 1995). If so, then different PIPkin isoforms, and hence the PtdInsP$_2$ pools they control, are likely to be subject to different regulatory mechanisms.

Several groups have investigated the regulation of PtdInsP$_2$ synthesis: product inhibition by PtdInsP$_2$ has been reported in several tissues (Van Rooijen *et al.*, 1985; Lundberg *et al.*, 1986; Pike and Arndt, 1988), whilst PtdInsP$_2$ production is stimulated when fibroblasts adhere to fibronectin (McNamee *et al.*, 1993), suggesting that integrin-mediated signals may activate PIPkin activity. EGF stimulates PtdInsP$_2$ synthesis in isolated A431 cell membranes (Payrastre *et al.*, 1990) and PIPkin activity co-immunoprecipitates with the EGF receptor from B82L cells (Cochet *et al.*, 1991), suggesting possible regulation by growth factors. Agonist-stimulated increases in PIPkin activity in the cytoskeletons of platelets (Grondin *et al.*, 1991) and A431 cells (Payrastre *et al.*, 1991) have also been reported and phorbol esters stimulate PtdInsP$_2$ production in platelets (Halenda and Feinstein, 1984). Finally,

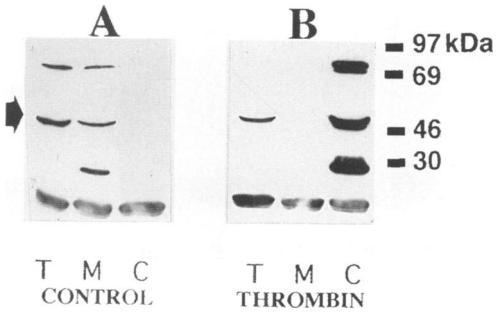

Fig. 1. Subcellular distribution of PIPkin C in resting and stimulated human platelets. Isolated platelets (~2×10^8 cells/sample) were stirred at 37°C in the absence (**A**) or presence (**B**) of thrombin (1 U/ml) for 10 min. The cells were lysed with Triton and the cytoskeleton (C), membrane skeleton (M) and Triton-soluble (T) fractions isolated. PIPkin C was solubilized from the entire cytoskeletal and membrane skeletal fractions with deoxycholate and immunoprecipitated from these and from 10% of the total Triton-soluble fractions. Immunoprecipitates were electrophoresed and Western blotted. PIPkin C (53 kDa, arrow) was detected immunologically. The two bands with approximate molecular weights of 30 and 80 kDa (which are recognized by three of the five anti-PIPkin C antibodies) have not been identified and will not be considered further. The band of 25 kDa corresponds to the light chain of the immunoprecipitating antibody. The heavy chain is not visible on this blot as the second antibody used is specific to the light chain. The positions of molecular weight markers are indicated.

G protein-stimulated PIPkin activity has been found in several tissues (Smith and Chang, 1989; Urumow and Wieland, 1990; Stephens *et al.*, 1993). A report from Chong *et al.* (1994) suggests that the small G protein rho is involved in regulating $PtdInsP_2$ synthesis in mouse fibroblast cell lines, whilst a PIPkin activity co-immunoprecipitates with the small G protein rac from Swiss 3T3 cell lysates (Tolias *et al.*, 1995). Interestingly, rac uncaps actin filament barbed ends and promotes actin filament growth in permeabilized platelets by a mechanism involving $PtdInsP_2$ production (Hartwig *et al.*, 1995). Despite this evidence for the existence of a variety of regulatory mechanisms, however, in none of these examples have the PIPkin isoforms involved been identified unequivocally.

The blood platelet provides a convenient tool for the investigation of cell signalling events. Not only is it already well characterized in terms of signal transduction, it also undergoes major structural reorganization in response to stimulation, permitting the study of cytoskeletal regulation. Earlier studies on platelets suggest that thrombin stimulation rapidly promotes increased $PtdInsP_2$ synthesis (Lassing and Lindberg, 1990; Racaud-Sultan *et al.*, 1993) and also increases PIPkin activity associated with the cytoskeleton (Grondin *et al.*, 1991). Furthermore, platelets contain high levels of PIPkin C (Divecha *et al.*, 1995). We have therefore used our panel of monoclonal antibodies against this isoform (Brooksbank *et al.*, 1993) to investigate its regulation and function in human platelets.

Results

To investigate whether changes in the intracellular distribution of PIPkin C contribute to the observed increase in cytoskeletal PIPkin activity in thrombin-stimulated platelets (Grondin *et al.*, 1991) we used our monoclonal antibodies to detect it in different platelet fractions. Figure 1A shows that, in unstimulated platelets, no PIPkin C immunoreactivity is present in the low speed Triton-insoluble fraction (the cytoskeleton), whereas ~95% is Triton soluble. The remaining PIPkin C is associated with the membrane skeleton, a Triton-insoluble network of short actin filaments and associated proteins underlying the plasma membrane, which sediments at high centrifugation speeds (Fox *et al.*, 1988, 1993).

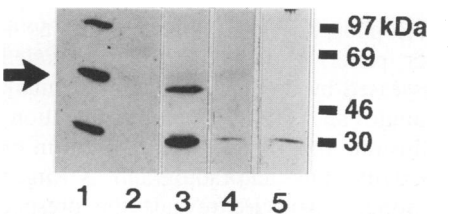

Fig. 2. Effect of stirring and RGDS peptide on PIPkin C translocation. Platelets were incubated at 37°C for 10 min under the following conditions: lane 2, no addition (stirred); lane 3, plus 1 U/ml thrombin (stirred); lane 4, plus 1 U/ml thrombin (unstirred); lane 5, plus thrombin and 200 μg/ml RGDS peptide (stirred). The cytoskeletons were electrophoresed, blotted and probed for PIPkin C (arrow). Lane 1 contains Triton-soluble material from unstimulated platelets as a positive control. The positions of molecular weight markers are indicated.

When platelets are stirred in the presence of thrombin (1 U/ml) a marked change occurs in the distribution of PIPkin C: after 10 min the enzyme is no longer detectable in the membrane skeleton, whereas ~25% of total PIPkin C is now present in the cytoskeleton (Figure 1B). A number of membrane skeletal proteins relocate to the cytoskeleton on aggregation (Asijee *et al.*, 1990; Fox *et al.*, 1993). However, although membrane skeletal PIPkin C may contribute to that appearing in the cytoskeleton, the larger proportion of total PIPkin C in the latter structure (Figure 1B) compared with the former (Figure 1A) demonstrates that much of the cytoskeletal enzyme must originate elsewhere.

To investigate the phenomenon of PIPkin C translocation to the cytoskeleton more fully, the experiments shown in Figure 2 were performed. Thrombin-stimulated translocation of PIPkin C to the cytoskeleton does not occur in the absence of stirring (lane 4), suggesting that platelet aggregation is required. Thrombin stimulation causes the secretion of fibrinogen and the conversion of the fibrinogen receptor, the integrin $\alpha_{2b}\beta_3$, to a high affinity state, allowing the secreted fibrinogen to bind to it (Shattil *et al.*, 1994). Stirring promotes the collision and consequent aggregation of the platelets via the fibrinogen–$\alpha_{2b}\beta_3$ complex, triggering integrin-mediated signals (Shattil *et al.*, 1994). In the absence of stirring, aggregation does not occur and the integrin-dependent signals are not activated. The finding that stirring is necessary for translocation of PIPkin C to the cytoskeleton of thrombin-stimulated platelets therefore implicates integrin-mediated signals in its instigation. This is confirmed by the ability of the peptide RGDS, which competes with fibrinogen for binding to $\alpha_{2b}\beta_3$ (Gartner and Bennett, 1985), to prevent the thrombin-stimulated translocation of PIPkin C to the cytoskeleton (Figure 2, lane 5).

To compare the observed translocation of PIPkin C to the cytoskeleton with the reported thrombin-stimulated increase in cytoskeletal PIPkin activity (Grondin *et al.*, 1991), we measured the ability of isolated platelet cyto-

skeletons to phosphorylate exogenous PtdIns 4-P. Figure 3A (upper panel) shows that the cytoskeletal PIPkin activity assessed by this method increases markedly over several minutes after stimulation of aggregation by thrombin. To illustrate this progressive increase in cytoskeletal PIPkin activity, this autoradiograph is slightly underexposed; longer exposure reveals the presence of low levels of PIPkin activity in the cytoskeleton of unstimulated platelets (not shown). The increase in cytoskeletal PIPkin activity is again dependent on aggregation, as it is prevented by RGDS peptide (Figure 3A, lower panel).

To determine whether any of the $PtdInsP_2$ produced by the cytoskeletons of thrombin-aggregated platelets is the PtdIns 3,4-P_2 isomer, which is not resolved from PtdIns 4,5-P_2 by the thin layer chromatography system used to separate phospholipids in this study, we deacylated and deglycerated the extracted cytoskeletal lipids and separated the head groups obtained using HPLC. The results (Figure 3B) clearly show that PtdIns 4-P phosphorylation by platelet cytoskeletons occurs exclusively in the 5 position under the experimental conditions used, confirming that the increase in $PtdInsP_2$ production by isolated cytoskeletons is due to an increase in PIPkin activity, rather than an increase in PI 3-kinase activity. Although PI 3-kinase activity is present in the cytoskeleton of aggregated platelets (Zhang *et al.*, 1992), under the assay conditions used here it does not cause significant production of PtdIns 3,4-P_2.

Figure 3C shows the time course over which PIPkin C translocates to the cytoskeleton in aggregating platelets. This is strikingly similar to that over which the PIPkin activity associated with the cytoskeleton increases (Figure 3A, upper panel), suggesting a possible causal connection between the two.

Although $PtdInsP_2$ is a phospholipid, it is not found exclusively in membranes: Fukami *et al.* (1994) have found that the cytoskeletons of Balb c/3T3 cells contain $PtdInsP_2$ bound to several proteins, including α-actinin and vinculin. To determine whether translocation of PIPkin C to the cytoskeleton and the accompanying increase in cytoskeletal PIPkin activity were associated with changes in cytoskeletal $PtdInsP_2$, we isolated cytoskeletons from Triton-lysed platelets that had been subjected to different conditions and measured their $PtdInsP_2$ contents. Figure 4A shows that cytoskeletons from unstimulated platelets contain a small quantity of $PtdInsP_2$ (138.8 ± 28 pmol/mg protein). Stimulation with thrombin in the absence of stirring causes a 3- to 6-fold increase in cytoskeletal protein content (Figure 4B). The $PtdInsP_2$ levels also rise by a similar amount, leaving the $PtdInsP_2$ content per unit protein at a similar level to that seen in unstimulated cells (Figure 4A). When the stimulated platelets are stirred, little further change in cytoskeletal protein content occurs (Figure 4B). However, a clear increase in the amount of $PtdInsP_2$ per unit protein is seen under these conditions; this is modest at 2 min (314.6 ± 119.3 pmol/mg), but large after 10 min (924.0 ± 220.0 pmol/mg). The dependence of this increase on stirring again suggests that it is controlled by integrin signalling, and this is once more confirmed by the ability of RGDS to prevent it (Figure 4A).

To confirm that the observed increase in $PtdInsP_2$ levels in the cytoskeleton does not merely reflect entrapment

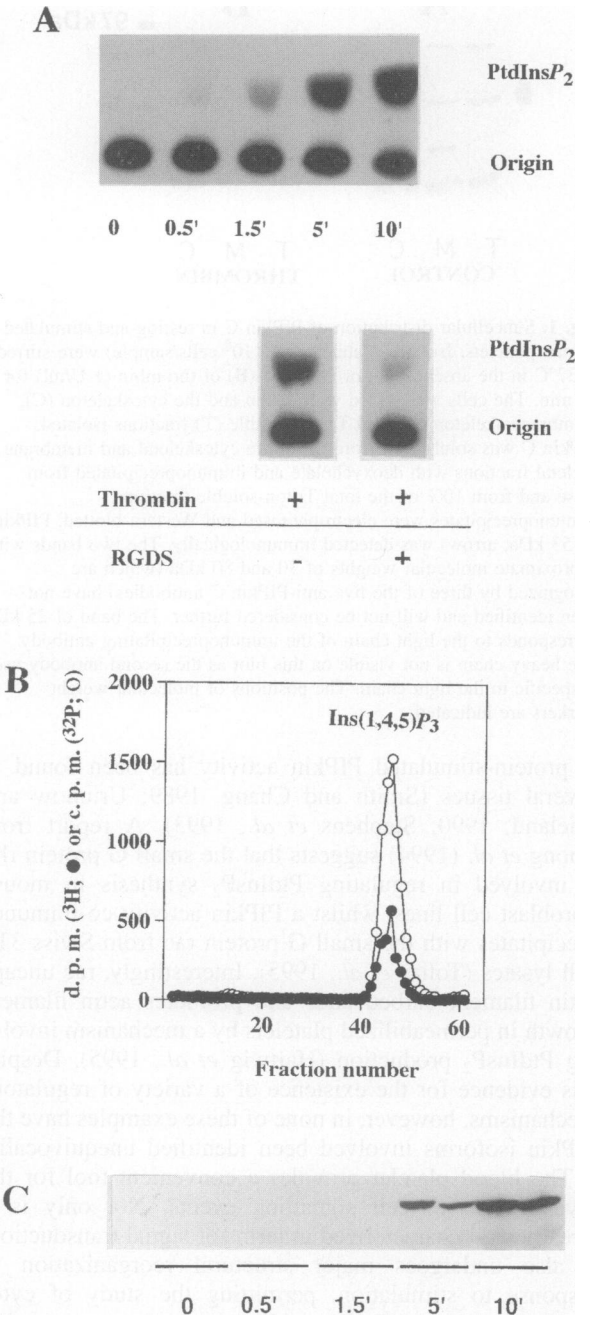

Fig. 3. Time course of increased cytoskeletal PIPkin activity and PIPkin C translocation. (**A**) (Upper panel) Platelets were stirred in the presence of thrombin (1 U/ml) for the indicated times. The cytoskeletons were then isolated and assayed for PIPkin activity. ^{32}P-Labelled lipids were separated by thin layer chromatography. The position of $PtdInsP_2$ is indicated. (Lower panel) Platelets were stirred for 10 min in the presence or absence of thrombin (1 U/ml). Some incubations with thrombin also contained RGDS peptide (200 μg/ml). The PIPkin activity associated with the cytoskeletons was then measured. The position of [^{32}P]$PtdInsP_2$ is indicated. (**B**) HPLC profile of ^{32}P-labelled inositol phosphates obtained from the phosphorylation of exogenous PtdIns 4-P by isolated platelet cytoskeletons obtained from platelets stirred with thrombin (1 U/ml) for 10 min at 37°C. All the ^{32}P-labelled products migrated with the [^{3}H]Ins 1,4,5-P_3 standard. Ins 1,3,4-P_3 migrates approximately eight fractions ahead of Ins 1,4,5-P_3 (not shown) and was not detected. (**C**) Time course of PIPkin C translocation to the cytoskeleton. Platelets were stirred in duplicate with thrombin (1 U/ml) for the indicated times. The cytoskeletons were electrophoresed, Western blotted and probed for PIPkin C.

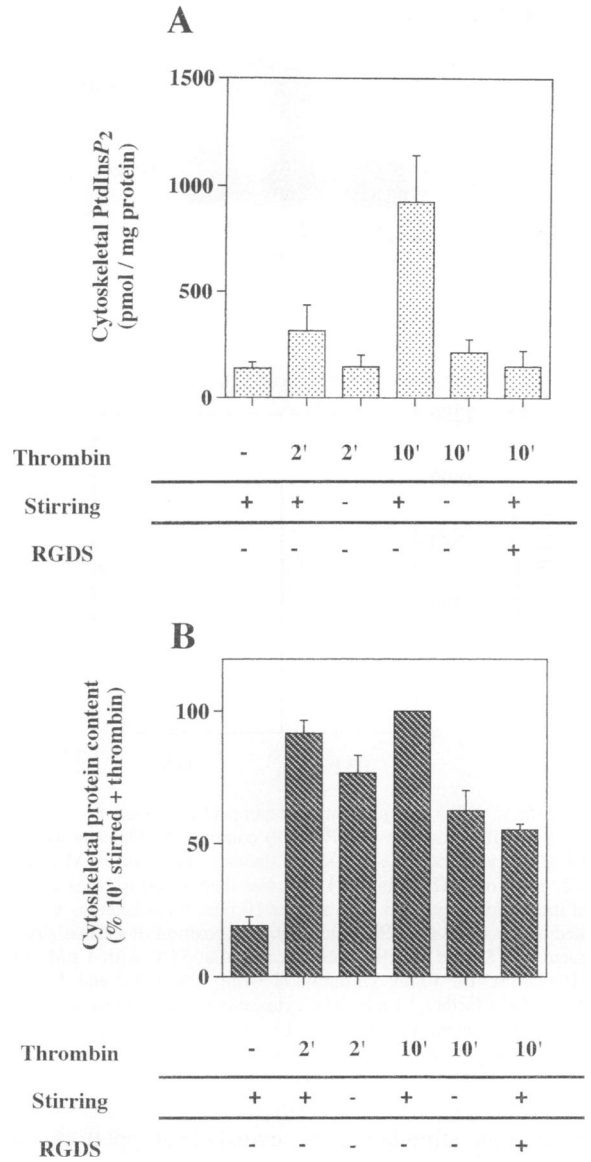

Fig. 4. Changes in cytoskeleton-associated $PtdInsP_2$ and protein content in thrombin-stimulated platelets. (**A**) Platelet samples were incubated in duplicate with or without thrombin (1 U/ml) or RGDS (200 μg/ml) for the indicated times in the presence or absence of stirring, as shown. The cytoskeletons were isolated and washed. One sample from each pair was then used to determine cytoskeletal protein content and the $PtdInsP_2$ content of the other measured. The results are means ± SEM of three to five independent experiments. (**B**) The cytoskeletal protein content from platelets exposed to the same conditions as in (A) is shown, expressed as a percentage of that from platelets stirred with thrombin for 10 min. Results are the means ± SEM of three to five independent experiments.

of $PtdInsP_2$-containing, Triton X-100-resistant membrane fragments in the cytoskeleton of large platelet aggregates (Zucker and Masiello, 1983; Livne *et al.*, 1988) we measured the total cytoskeletal lipid (measured as lipid phosphate) under different conditions. Although the amount of phospholipid associated with the cytoskeleton increases ~10-fold in platelet aggregates compared with unstimulated cells, it does so with an entirely different (faster) time course than that of the increase in cytoskeletal $PtdInsP_2$ levels (not shown). In fact, the lipid levels in the cytoskeleton reach 70% of their maximal value within 1 min of stimulation and are maximal within 5 min, clearly demonstrating that retention of membrane fragments cannot directly be responsible for the increase in cytoskeleton-associated $PtdInsP_2$. It remains possible, however, that the entrapment of membrane fragments may reflect formation of regions of close association between cytoskeleton and plasma membrane during platelet aggregation and that PtdIns 4-P present within such regions may subsequently be phosphorylated by cytoskeletal PIPkin C. The $PtdInsP_2$ so produced could either remain in the membrane or, alternatively, bind to cytoskeletal proteins.

The aggregation-dependent increase in $PtdInsP_2$ associated with the cytoskeleton (Figure 4A) again occurs over a similar period to that over which the increase in cytoskeletal PIPkin activity is observed (Figure 3A) and over which PIPkin C translocates to the cytoskeleton (Figure 3C), once more suggesting a causal connection between these events. Much of the increase in cytoskeletal protein content occurs irrespective of whether the platelets aggregate (Figure 4B), whilst PIPkin C translocation (Figures 2 and 3C) and the integrin-dependent increases in cytoskeletal $PtdInsP_2$ levels (Figure 4A) and PIPkin activity (Figure 3A) are not only aggregation dependent, but also occur more slowly than the stimulation-dependent increase in cytoskeletal protein (which is complete within 2 min of stimulation; see Figure 4B). This suggests that translocation of PIPkin C to the cytoskeleton does not merely reflect non-specific entanglement of the enzyme in newly formed cytoskeletal structures, but that, on the contrary, the process is highly specific.

The protein phosphatase 1/2A inhibitor okadaic acid (OA) has been reported to inhibit the platelet aggregation stimulated by 0.1 U/ml thrombin (Higashihara *et al.*, 1992). In our hands, OA does not prevent aggregation in response to a higher thrombin concentration (1 U/ml), although it significantly delays the process (Figure 5A). This is not inconsistent with the findings of Higashihara *et al.* (1992), who stated that the effects of OA were reduced at higher thrombin concentrations. Furthermore, Lerea (1991) has also reported that the inhibitory effects of OA on platelet secretion were overcome at high agonist doses.

Although under the experimental conditions used here OA pretreatment does not prevent platelet aggregation, we do find that it abolishes translocation of PIPkin C to the cytoskeleton (Figure 5B): under these conditions the OA-pretreated platelets aggregate within 10 min of stimulation and the gross changes in cytoskeletal protein content induced by thrombin are complete within this time (compare Figure 5C, lanes 2–4), but no PIPkin C is detected in the cytoskeleton even after 20 min stimulation (Figure 5B).

The non-receptor tyrosine kinase $pp60^{c\text{-}src}$ also translocates to the platelet cytoskeleton during aggregation (Grondin *et al.*, 1991; Fox *et al.*, 1993; Dash *et al.*, 1995a). Translocation requires $pp60^{c\text{-}src}$ tyrosine kinase activity (Schoenwaelder *et al.*, 1994), but nothing more is known about the translocation mechanism. To test whether a similar mechanism might underlie translocation of $pp60^{c\text{-}src}$ and PIPkin C to the cytoskeleton we investigated the effects of OA pretreatment on the former process. Figure 5D shows that, as with PIPkin C, OA pretreatment abolishes $pp60^{c\text{-}src}$ translocation to the cytoskeleton in response to thrombin-stimulated aggregation and that even

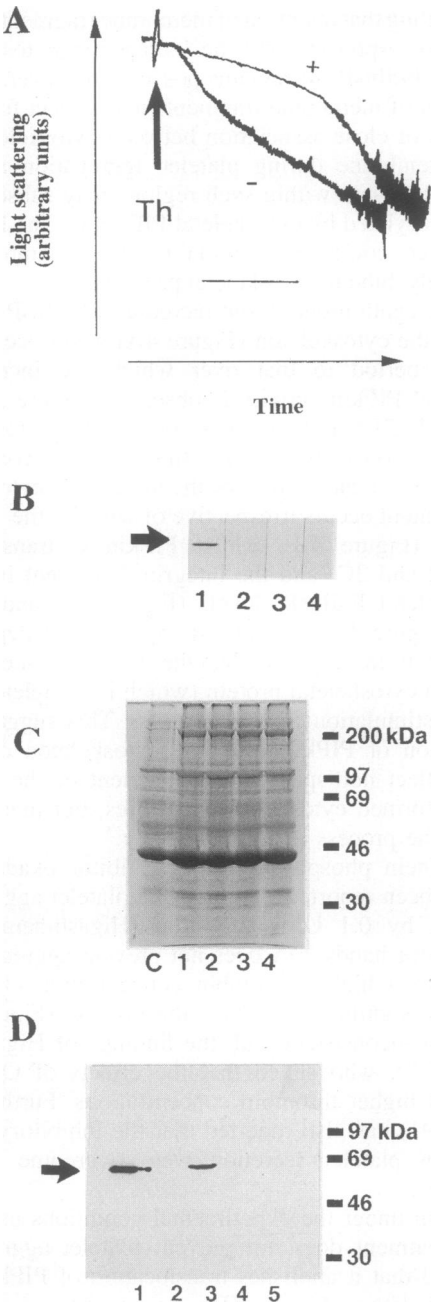

Fig. 5. Okadaic acid inhibits cytoskeletal translocation of PIPkin C and pp60$^{c\text{-}src}$. (**A**) Platelets were stirred at 37°C in the presence (+) or absence (–) of 1 μM OA for 5 min. Thrombin (1 U/ml) was then added (arrow) and the resulting aggregation followed. The bar denotes an interval of 1 min. (**B**) Stirred platelets were incubated for 5 min at 37°C with 0.2% DMSO (lane 1) or 2 μM OA (lanes 2–4), followed by thrombin for a further 10 (lanes 1 and 2), 15 (lane 3) or 20 (lane 4) min. Cytoskeletons were electrophoresed, Western blotted and immunoprobed for PIPkin C (arrow). (**C**) Coomassie-stained gel of platelet cytoskeletons treated as in (B) (lane C contains the cytoskeleton from an equal number of unstimulated platelets for comparison). The positions of molecular weight markers (in kDa) are indicated. (**D**) Stirred platelets were incubated at 37°C in the presence of 0.2% DMSO (lanes 2 and 3) or 2 μM OA (lanes 4 and 5) for 5 min. Thrombin (1 U/ml) was then added to lanes 3–5 and incubation continued for a further 10 (lanes 2–4) or 20 (lane 5) min. The cytoskeletons were isolated, electrophoresed, Western blotted and the presence of pp60$^{c\text{-}src}$ (arrow) detected immunologically. Lane 1 contains whole platelet lysate as a positive control. The positions of molecular weight markers (in kDa) are indicated.

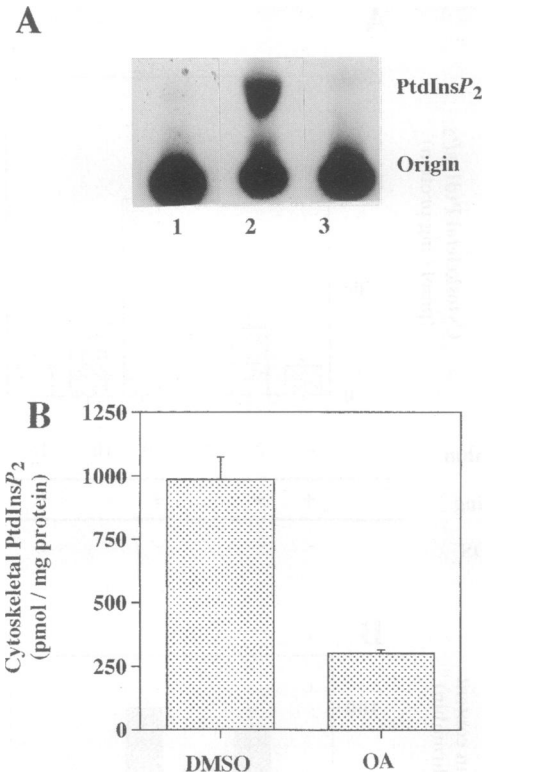

Fig. 6. Effects of OA on aggregation-dependent increases in cytoskeletal PIPkin activity and PtdInsP_2 content. (**A**) Platelets were stirred in the presence of 0.2% DMSO (lanes 1 and 2) or 2 μM OA (lane 3) for 5 min. Thrombin (1 U/ml) was then added to lanes 2 and 3 and incubation continued for a further 10 min. Cytoskeletons were isolated and assayed for PIPkin activity. The position of PtdInsP_2 is indicated. (**B**) Stirred platelets were incubated at 37°C with 1 μM OA or 0.1% DMSO for 5 min. Thrombin (1 U/ml) was added and the cells incubated for a further 10 min. The cytoskeletons were isolated and their PtdInsP_2 content measured. Incubations were performed in triplicate. Results (mean ± SEM) are from a single experiment, representative of two.

after 20 min stimulation no cytoskeletal pp60$^{c\text{-}src}$ can be detected.

We next investigated whether OA pretreatment was also able to abolish the aggregation-dependent increases in the levels of PtdInsP_2 associated with the cytoskeleton and in cytoskeletal PIPkin activity. Figure 6 shows that this is indeed the case. Figure 6A shows that OA pretreatment abolishes the increase in cytoskeletal PIPkin activity associated with aggregation. Note that the OA concentration in all samples was adjusted to 2 μM after lysis and the cytoskeletons were extensively washed prior to assay, suggesting that this abolition is not due to a direct effect of the inhibitor on cytoskeletal PIPkins. Figure 6B demonstrates that the aggregation-dependent increase in cytoskeletal PtdInsP_2 levels is also abolished by OA pretreatment, again demonstrating that prevention of PIPkin C translocation to the cytoskeleton also abolishes the integrin-dependent increase in PtdInsP_2 bound to this structure.

Discussion

Our results demonstrate that the cytoskeletal fractions of detergent-lysed platelets contain a pool of PtdInsP_2, the size of which increases in response to the integrin-mediated

signalling events associated with platelet aggregation. Integrin-mediated signals also cause a marked increase in cytoskeletal PIPkin activity and a concomitant translocation of PIPkin C to the cytoskeleton with a similar time course to the observed increase in $PtdInsP_2$ levels, strongly suggesting that these events are linked. Further support for this idea comes from the finding that all three phenomena are abolished by OA pretreatment. Thus, whilst we cannot rule out a contribution from other, immunologically distinct PIPkin isoforms, it is likely that signals mediated via the integrin $\alpha_{2b}\beta_3$ cause an increase in cytoskeletal $PtdInsP_2$ levels which is (at least in part) mediated via a change in the subcellular distribution of PIPkin C. Integrin-mediated signals are known to increase $PtdInsP_2$ production in C3H 10T1/2 fibroblasts (McNamee *et al.*, 1993) and integrin-containing focal adhesion complexes from capillary endothelial cells contain a PIPkin activity (Plopper *et al.*, 1995), although in neither case has the PIPkin isoform involved been identified. As integrin signalling is still imperfectly understood, it is possible that PIPkin C may be commonly involved in the signalling cascades initiated by this family of molecules.

In agreement with Grondin *et al.* (1991), we find that cytoskeletons from unstimulated platelets contain a small amount of PIPkin activity, although the absence of PIPkin C immunoreactivity suggests that a different PIPkin isoform is responsible. It is probable that this activity is responsible for the small amount of $PtdInsP_2$ associated with the cytoskeletons of unstimulated platelets. However, our results differ from those of Grondin *et al.* in that these authors detected a very rapid increase in the levels of cytoskeletal PIPkin activity in response to thrombin, which reached a plateau within 60 s. This is clearly much more rapid than the increase in cytoskeletal PIPkin activity presented in Figure 3A. However, the conditions of our assay differ from those used by Grondin and co-workers: as our experiments were aimed at assessing the contribution of PIPkin C to cytoskeletal PIPkin activity, we used an ATP concentration of 5 μM (close to the K_m of purified PIPkin C; Divecha *et al.*, 1992) and a PtdIns 4-P concentration of 5 μM. The ATP and PtdIns 4-P concentrations used by Grondin *et al.* were both 500 μM. The early increase in cytoskeletal PIPkin activity detected by Grondin *et al.* is clearly not caused by PIPkin C, as this isoform does not translocate to the cytoskeleton until later in the aggregation process (Figure 3C), nor is it likely to contribute to the integrin-dependent increase in cytoskeleton-associated $PtdInsP_2$, as this is also a later event (Figure 4A). Instead, it is likely to be produced by a different PIPkin isoform that is subject to different regulatory processes and which produces $PtdInsP_2$ for other purposes.

In addition to PIPkin C, PI 3-kinase and $pp60^{c\text{-}src}$, many other signal transduction molecules also translocate to the cytoskeleton in aggregating platelets. These include the tyrosine kinases $pp62^{c\text{-}yes}$, $pp72^{c\text{-}syk}$ and $pp125^{FAK}$ (Fox *et al.*, 1993; Clark *et al.*, 1994; Dash *et al.*, 1995a), ras GTPase activating protein (Fox *et al.*, 1993) and the low molecular weight G proteins Rap 1b, Rap 2b, CDC42Hs and rac (Torti *et al.*, 1994; Dash *et al.*, 1995a,b). Additionally, cytoskeletal levels of phospholipase C, PtdIns 4-kinase and DAG kinase activities all increase (Grondin *et al.*, 1991), although actual translocation of these enzymes to the cytoskeleton has not been demonstrated. It is interesting to note that rac and rho both translocate to the platelet cytoskeleton in response to thrombin (Zhang *et al.*, 1993; Dash *et al.*, 1995b) (rho translocation apparently being integrin independent), where they may conceivably be involved in regulating PIPkins (Chong *et al.*, 1994; Tolias *et al.*, 1995).

Although the reason for the translocation of these proteins to the cytoskeleton is obscure, it may be involved in comparatively late, integrin-mediated processes such as fibrin clot retraction (Schoenwaelder *et al.*, 1994). The delayed translocation of PIPkin C to the cytoskeleton (compare the rate of platelet aggregation in Figure 5A with the time course of PIPkin C translocation in Figure 3C) is consistent with this idea. The mechanism(s) by which the cytoskeletal translocation of signalling proteins is accomplished remains obscure, but some insight may be given by our finding that PIPkin C and $pp60^{c\text{-}src}$ translocation are both abolished by OA in the continued presence of platelet aggregation. This suggests that the translocation of both enzymes may be controlled by a common mechanism and that changes in protein phosphorylation are involved. Unfortunately, however, it is not possible to determine the specific point at which OA acts to block translocation, as the inhibitor causes marked changes in the phosphorylation of many platelet proteins and also prevents thrombin-stimulated phosphorylation of certain others (Higashihara *et al.*, 1992). The recent cloning of PIPkin C has revealed the presence of a proline-rich region constituting a putative SH_3 binding domain (Boronenkov and Anderson, 1995; Divecha *et al.*, 1995). It is possible that this region may be involved in translocation of PIPkin C to the cytoskeleton, but confirmation of this hypothesis must await the identification of proteins that interact with it.

The function of the cytoskeleton-associated $PtdInsP_2$ pool controlled by PIPkin C is unclear, as is the question of whether it is bound directly to cytoskeletal proteins or remains in associated membrane fragments. It cannot be involved in mediating thrombin-induced actin polymerization, as this occurs before the major increase in cytoskeleton-associated $PtdInsP_2$ levels and is largely independent of platelet aggregation (see Figure 4). Instead, it is again more likely to be involved in late, integrin-dependent processes such as clot retraction. It is possible that the cytoskeleton-associated $PtdInsP_2$ pool provides the substrate for phospholipase C or PI 3-kinase, both of which are active in the cytoskeleton after aggregation (Grondin *et al.*, 1991; Zhang *et al.*, 1992). Alternatively, it may directly mediate changes in the cytoskeleton via interactions with actin binding proteins. In contrast to the increase in cytoskeleton-associated $PtdInsP_2$ levels reported here, the amount of $PtdInsP_2$ bound to the cytoskeleton of Balb c/3T3 cells (detected by an anti-$PtdInsP_2$ monoclonal antibody) decreases on stimulation with PDGF (Fukami *et al.*, 1994), possibly due to $PtdInsP_2$ hydrolysis by phospholipase Cγ. It is possible that this discrepancy reflects cell type-specific differences in the role of the cytoskeleton-associated $PtdInsP_2$ pool, perhaps associated with differences in cytoskeletal remodelling in response to different stimuli. $PtdInsP_2$ in Balb c/3T3 cytoskeletons is bound, amongst other proteins, to α-actinin and vinculin (Fukami *et al.*, 1994); it will be

interesting to identify the cytoskeletal proteins to which it binds in platelets.

Whatever its function is ultimately determined to be, the discovery of this novel integrin-regulated pool of $PtdInsP_2$ supports the idea that discrete, functionally distinct pools of this important lipid are present within cells. The finding that PIPkin C is involved in its regulation constitutes the first demonstration of a specific role for this isoform in a defined subcellular context.

Materials and methods

Materials

Horseradish peroxidase (HRP)-linked anti-rat antibodies, RGDS peptide and Sepharose CL-4B were from Sigma. [γ-^{32}P]ATP, [^{3}H]Ins 1,4,5-P_3, HRP-linked anti-mouse antibodies, prestained protein markers and enhanced chemiluminescence (ECL) reagents were from Amersham International. Nitrocellulose was from Anderman and Co., protein G–Sepharose was from Pharmacia. Anti pp60$^{c\text{-}src}$ monoclonal antibody 327 was a generous gift from Dr Joan Brugge. OA was from Calbiochem or Sigma. Silica-coated glass thin layer chromatography plates were from Merck, autoradiography film was from Amersham (for ECL) or GRI (for ^{32}P detection). Emulsifier Scintillator Plus scintillation fluid was from Packard. All other reagents were of analytical grade.

Preparation of platelets

Blood (50 ml) from healthy human volunteers who denied having taken aspirin within the previous 48 h was mixed with the anticoagulant acid citrate dextrose (97 mM sodium citrate, 78 mM citric acid, 110 mM D-glucose). Platelet-rich plasma was obtained by centrifugation (20 min, 150 *g*) and removal of contaminating red and white blood cells. EGTA (1 mM) was added and the platelets sedimented by centrifugation at 900 *g* for 15 min. The pellet was resuspended in platelet buffer (10 mM HEPES, pH 7.4, 10 mM glucose, 1 mM $MgSO_4$, 145 mM NaCl, 5 mM KCl) and washed by gel filtration on a Sepharose CL-4B column. The protein concentration of the platelet suspension was adjusted to ~0.5 mg/ml.

Cytoskeleton and membrane skeleton isolation

Platelets were incubated at 37°C in the presence of 2 mM $CaCl_2$, with or without stirring. Agonists or inhibitors were added at intervals and the cells were lysed with an equal volume of platelet buffer containing 2% Triton X-100, 10 mM EDTA, 6 mM EGTA, 100 mM NaF, 200 μM Na_3VO_4, 2 mM PMSF and 1 μg/ml leupeptin. Cytoskeletons were isolated by centrifugation at 10 000 *g* (10 min, 4°C), washed twice with 1 ml ice-cold platelet buffer containing 1% Triton X-100, 2 mM EGTA and 5 mM EDTA and once with the same buffer without detergent. In some experiments, the cytoskeletons were then boiled in SDS–PAGE sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 5% 2-mercaptoethanol), electrophoresed and Western blotted. In others, cytoskeletal PIPkin C was solubilized with 1% sodium deoxycholate in platelet buffer with added protease inhibitors and immunoprecipitated with the anti-PIPkin C monoclonal antibody MAC 334 coupled to protein G–Sepharose beads for 2 h at 4°C with mixing. Immunoprecipitates were washed three times with Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5, 140 mM NaCl), drained, then sonicated and boiled in 20 μl SDS–PAGE sample buffer prior to electrophoresis.

Membrane skeletons were isolated as described by Fox *et al.* (1988). Briefly, the 10 000 *g* supernatant was centrifuged at 100 000 *g* for 3 h at 4°C. The pellet was washed twice in 1 ml ice-cold platelet buffer, 2 mM EGTA and the membrane skeletal PIPkin C was solubilized and immunoprecipitated with MAC 334 as described above. The Triton X-100-soluble fraction of the lysate was prepared for electrophoresis by boiling in SDS–PAGE sample buffer.

SDS–PAGE and Western blotting

Samples were electrophoresed on 10% acrylamide gels incorporating a 3.8% stacking gel and transferred electrophoretically to supported nitrocellulose membranes. PIPkin C was detected immunologically using a mixture of the anti-PIPkin C antibodies MAC 334, MAC 341 and MAC 344, an HRP-conjugated mouse anti-rat second antibody and ECL. pp60$^{c\text{-}src}$ was detected using mAb 327, an HRP-conjugated sheep anti-mouse second antibody and ECL. Antibodies were presented in a mixture of 5% non-fat dried milk in TBS containing 0.05% Tween-20.

Cytoskeletal PIPkin assay

Cytoskeletons were isolated as described above except that only the initial wash contained Triton X-100. Washed cytoskeletons were incubated at 30°C for the indicated times in the presence of PtdIns 4-P (5 μM) and ATP (5 μM containing 25 μCi/ml [γ-^{32}P]ATP). Reaction was stopped by the addition of 1 ml 1:1 (v:v) chloroform:methanol and the phases split by the addition of 250 μl 2.4 N HCl. The lower phase was washed once with theoretical upper phase (1 N HCl:methanol:chloroform, 48:47:3 by vol.), extracted, dried in a vac-fuge, redissolved in chloroform and spotted onto a silica gel-coated thin layer chromatography plate that had been dipped into 1% potassium oxalate solution and activated at 110°C for 1 h. Lipids were resolved using chloroform:methanol:ammonia solution:water (45:35:2:8 by vol.) as solvent. $PtdInsP_2$ was identified by the inclusion of a standard. Radioactive $PtdInsP_2$ was visualized by exposure of the plate to autoradiography film.

$PtdInsP_2$ mass assay

Lipids were extracted from washed platelet cytoskeletons by sonication in the presence of 1 ml 1:1 (v:v) chloroform:methanol. Aliquots of 250 μl 2.4 N HCl were added and the mixture vortexed thoroughly, centrifuged briefly and the lower phase dried in a vac-fuge. The samples were deacylated by incubation with monomethylamine reagent (prepared according to Clarke and Dawson, 1981) for 30 min at 52°C, dried down, dissolved in water, washed with a 20:4:1 (by vol.) butan-1-ol:petroleum ether:ethyl formate to remove fatty acids, dried down again and deglycerated. Samples were incubated with 200 μl 10 mM sodium periodate for 20 min at room temperature in the dark, after which 10 μl ethylene glycol (1:17 v:v in water) were added and incubation continued for a further 30 min. Next, 750 μl 1% aqueous dimethylhydrazine (pH 4.5 with formic acid) was added and the samples incubated for 4 h at room temperature before being dried down. The Ins 1,4,5-P_3 content of the samples, and hence their original $PtdInsP_2$ content, was determined by displacement of bound [^{32}P]Ins 1,4,5-P_3 from a preparation of adrenal cortical microsomes using the method of Palmer and Wakelam (1990). The [^{32}P]Ins 1,4,5-P_3 used in this assay was prepared by deacylation and deglyceration of $PtdInsP_2$ that had been labelled with ^{32}P in the 5 position. This in turn was prepared using [γ-^{32}P]ATP, pure PtdIns 4-P and purified PIPkin C.

Platelet aggregation

Platelet aggregation was assessed using a Chromo Log Corporation dual aggrometer, observing the decrease in light scattering over time after addition of thrombin to stirred platelets maintained at 37°C in the presence of 2 mM $CaCl_2$.

HPLC analysis of cytoskeletal $PtdInsP_2$

Cytoskeletons were prepared from stirred platelets incubated with thrombin (1 U/ml) for 10 min at 37°C as described above. Lipids were extracted, deacylated and deglycerated as described. The inositol phosphate samples obtained were filtered through a 0.45 μm filter and applied to a Partisil 10SAX HPLC column in the presence of ~2000 d.p.m. [^{3}H]Ins 1,4,5-P_3 as standard. The column was washed with 0.34 M NaH_2PO_4, pH 3.7, and any inositol trisphosphates present were eluted isocratically with 0.55 M NaH_2PO_4, pH 3.7. Fractions (0.25 ml) were collected and counted for ^{3}H and ^{32}P in 1 ml methanol and 3 ml scintillation fluid.

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