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Thrombin Receptor Ligation and Activated Rac Uncap Actin Filament Barbed Ends through Phosphoinositide Synthesis in Permeabilized Human Platelets

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Summary

Cells respond to diverse external stimuli by polymerizing cytoplasmic actin, and recent evidence indicates that GTPases can specify where this polymerization takes place. Actin assembly in stimulated blood platelets occurs where sequestered monomers add onto the fast-growing (barbed) ends of actin filaments (F-actin), which are capped in the resting cells. We report that D3 and D4 polyphosphoinositides, PI(4)P, PI(4,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3, uncap F-actin in resting permeabilized platelets. The thrombin receptor-activating peptide (TRAP), GTP, and GTPγS, but not GDPβS, also uncap F-actin in permeabilized platelets. GDPβS inhibits TRAP-induced F-actin uncapping, and PI(4,5)P_2 overcomes this inhibition. Constitutively active mutant Rac, but not Rho, activates uncapping of F-actin. PI(4,5)P_2-binding peptides derived from gelsolin inhibit F-actin uncapping by TRAP, Rac, and GTPγS. TRAP and Rac induce rapid PI(4,5)P_2 synthesis in permeabilized platelets. The findings establish a signaling pathway for actin assembly involving Rac in which the final message is phosphoinositide-mediated F-actin uncapping.

Introduction

Human platelets are tiny oval discs, 7 fl in volume, that circulate in the blood at a concentration of 2.5 × 10^8/ml. They are subject to stimulation by numerous agents generated by tissue injury, following which they undergo a profound shape transformation. First, the disc expands into a sphere, and then it projects two types of surface protrusions, flat lamellae and finger-like filopodia. The lamellae adhere to injured surfaces to plug vascular leaks, and the filopodia bind fibrin strands and other platelets to form a three-dimensional blood clot. These reactions are responsible for physiological hemostasis and also for thromboses associated with pathological states such as myocardial infarctions, peripheral vascular insufficiency, and strokes.

Remodeling of the actin filament scaffolding that holds the resting platelet in the discoid configuration is responsible for the cellular shape change, and the biochemistry and morphology of the actin remodeling have been the subject of much investigation (Fox, 1993; Fox et al., 1984; Hartwig, 1992; Nachmias et al., 1993). The polymeric actin cytoskeleton of the resting platelet consists of 2000 actin filaments cross-linked by the actin gelation factor ABP-280 (platelet filamin) and utilizes 40% of the 0.5 mM actin contained within the cell. The remainder of the actin is largely in the form of individual monomeric subunits, bound as 1:1 complexes to a small polypeptide (thymosin β4) or to profilin. As presently conceptualized, thymosin β4 inhibits the spontaneous nucleation of monomeric actin that is required for polymerization (Safer et al., 1990). Based on biochemical data, however, the fast-growing ends of actin filaments (conventionally designated as barbed in reference to arrowheads conferred by bound myosin head domains) have a higher affinity for actin monomers than thymosin β4, and profilin–actin complexes can add directly to the barbed end but not to the pointed end of filaments. Therefore, thymosin β4 can keep 60% of the total platelet actin unpolymerized only if the actin filament barbed ends in the cytoskeleton of the resting platelet are blocked ("capped") (Nachmias et al., 1993; Pantaloni and Carlier, 1993). The barbed ends of actin filaments in detergent-permeabilized resting platelets do not accommodate addition of actin subunits, consistent with these ends being capped, but barbed ends of actin filaments do become available to nucleate actin assembly following exposure of the cells to stimuli that elicit the platelet shape change (Hartwig, 1992; Nachmias et al., 1993).

One interesting question concerns the signal transduction mechanisms that regulate the actin remodeling in the activating platelet. Strong evidence indicates that calcium is required for the disc-to-sphere transformation that precedes actin polymerization and for the protrusion of flat lamellae, because the nature of the shape change and the extent of exposure of cryptic barbed ends depend on the ability of the platelet to increase its intracellular calcium concentration. Following platelet stimulation, the intracellular calcium rises from the basal nanomolar to micromolar levels (Davies et al., 1989). The source of calcium in this reaction is both extracellular and intracellular. Release of calcium from intracellular stores depends on the hydrolysis of phosphatidylinositol (4,5)bisphosphate (PI(4,5)P_2) by phospholipase Cβ to generate inositol trisphosphate, a reaction dependent upon a pertussis toxin–sensitive heterotrimeric GTPase (Brass et al., 1986). In this setting, the usual disc-to-sphere transformation takes place, several hundred barbed actin filament ends uncap, and the platelet extrudes lamellae and filopodia. An intermediate step in this reaction is a marked shortening of the actin filaments in the resting platelets, best explained by a calcium-

dependent actin filament–severing reaction mediated by calcium-activated actin filament–rupturing proteins, such as gelsolin, that reside within platelets (Hartwig, 1992). In support of this mechanism, platelets genetically devoid of gelsolin do not demonstrate the actin filament–shortening phenomenon (Witke et al., 1995). A permeant calcium-chelating agent that prevents the normal calcium transient and should therefore inhibit calcium-dependent actin filament–severing proteins has the same effect (Hartwig, 1992). Platelets lacking gelsolin or unable to raise intracellular calcium when activated extend filopodia but not lamellae.

Although calcium affects actin filament assembly, it does not mediate uncapping per se. In fact, micromolar calcium is required for actin filament barbed end capping by proteins of the gelsolin family in vitro, and although removal of calcium reverses capping by some members of this class of proteins, such as CapG and villin, it does not have this effect on others, including gelsolin or its homolog severin, or on an ubiquitous structurally unrelated barbed end capping protein of muscle and nonmuscle cells (Heiss and Cooper, 1991). Therefore, although platelets lacking gelsolin or prevented from increasing cytoplasmic free calcium following stimulation fail to sever their actin filaments, they do uncap them, and the uncapped barbed ends elongate extensively. Consistent with their inability to make additional barbed ends by severing, however, the number of uncapped barbed ends in these platelets is markedly diminished compared with gelsolin-containing or unchelated platelets (Hartwig, 1992; Witke et al., 1995).

The only physiological agents identified that remove barbed end capping proteins from the filament ends in vitro are the D4 polyphosphoinositides, phosphatidylinositol 4-phosphate (PI(4)P) and PI(4,5)P_2 (Janmey, 1994; Janmey and Stossel, 1989; Heiss and Cooper, 1991). Evidence that these phospholipids mediate barbed end uncapping in platelets or other cells, however, has thus far only been correlative. Thrombin stimulation of platelets initially diminishes by about 30% the high (200 μM) concentration of PI(4,5)P_2 found in resting cells. Within 15–30 s, however, PI(4,5)P_2 concentration rises to 40% over the resting baseline (Agranoff et al., 1983; Grondin et al., 1991). This overshoot provides the cell with an additional ~50 μM PI(4,5)P_2. The fall in phosphoinositides parallels the time course of the calcium rise and a transient appearance of gelsolin–actin complexes in extracts of stimulated platelets (Lind et al., 1987). The gelsolin–actin complexes then dissociate as the phosphoinositide level recovers in conjunction with a net increase in polymerized actin. Some (Apgar, 1995; Carson et al., 1992; Chaponnier et al., 1987) but not all (Dadabay et al., 1991) studies with other cell types documented similar temporal correlations between D4 phosphoinositide metabolism and actin assembly. Biosynthesis of D3 phosphoinositides also accompanies platelet activation (Gaudette et al., 1993; Grondin et al., 1991; Kucera and Rittenhouse, 1990; Sultan et al., 1991; Zhang et al., 1992, 1993), although inhibition of phosphatidylinositol 3-kinase activity has no detectable effect on barbed end exposure or net actin assembly in thrombin-stimulated platelets (Kovacsovics et al., 1995). If phosphoinositides uncap actin filaments in activated platelets, then the D4 class of these phospholipids is sufficient for most of this activity. Therefore, we have focused on the role of the D4 phosphoinositides in barbed end uncapping in these cells.

To obtain direct evidence for phosphoinositide-mediated barbed end uncapping in platelets and to begin to trace the signal transduction pathways linking surface stimulation to this uncapping event, we have taken advantage of two recent methodological advances. One is the development of a permeabilization scheme (described in this paper) that prepares platelets capable of uncapping barbed ends in response to stimulation of the thrombin receptor, of synthesizing phosphoinositides, and into which signal transduction intermediates and inhibitors can be delivered. The second is the availability of specific high affinity phosphoinositide-binding peptides derived from gelsolin (Janmey et al., 1992) that can be used to probe the role of phosphoinositides in the signal transduction mechanism.

Results

Relationship among Exposure of Barbed Actin Filament Ends, Actin Assembly, and Mass Content of D4 Phosphoinositides Following Thrombin Receptor Perturbation in Intact Platelets

Resting platelets have ~70 exposed barbed ends per cell, representing less than 4% of all the barbed ends present in a resting platelet (Hartwig, 1992). After activation of platelets by 10 μM thrombin receptor–activating peptide (TRAP), the number of exposed barbed filament ends rapidly increases (Figure 1). Approximately 280 free barbed ends appear 15 s after TRAP activation, increasing to a maximal value of ~560 after 60 s. Therefore, after maximal activation, 20%–25% of the total barbed filament ends

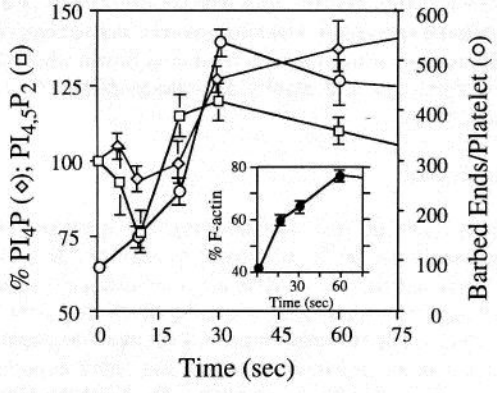

Figure 1. Temporal Relationship among D4 Polyphosphoinositide Changes, Exposure of Barbed Filament Ends, and Actin Assembly in Platelets Activated with 10 μM TRAP

Samples were simultaneously assayed for barbed filament ends (open circles), F-actin content (closed circles, inset), and the D4 polyphosphoinositides, PI(4)P (diamonds) and PI(4,5)P_2 (squares).

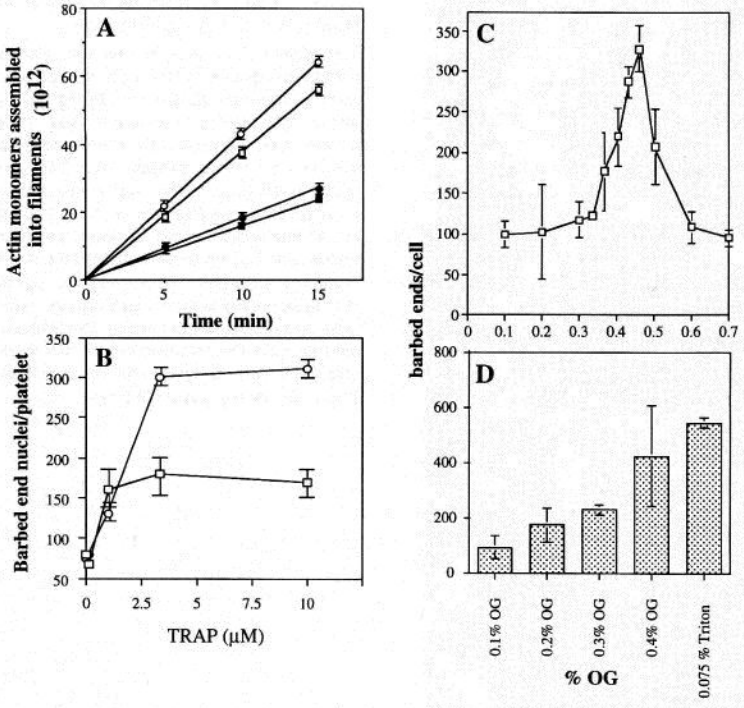

Figure 2. TRAP Uncaps Actin Filaments in Platelets Permeabilized with OG

(A) The ability of platelet lysates to stimulate the rate of pyrene–actin assembly in vitro was determined. Resting cells (closed circles) or cells activated with 10 μM TRAP for 1 min (open circles) were extracted with 0.4% OG. Resting cells permeabilized for 2 min were activated using 10 μM TRAP for 1 min prior to addition of pyrene–actin (open squares). Actin assembly in TRAP-activated cells was predominantly from the barbed ends of filaments as defined by cytochalasin B inhibition (closed squares). The rate of actin assembly in the presence and absence of cell lysates was determined, and the number of exposed barbed ends was calculated. They are 360 ± 42 for TRAP-activated and then permeabilized cells, 315 ± 32 for permeabilized and then TRAP-treated cells, and 90 ± 25 for resting cells (mean ± SD, n = 3).

(B) Comparison of the TRAP dose responses in intact and permeabilized cells. Platelets were stimulated with TRAP for 1 min prior to permeabilization (open circles) or permeabilized using 0.4% OG for 2 min and then treated with TRAP (squares).

(C) Relationship of the amount of detergent used to permeabilize the cells and the retention of TRAF-mediated filament end exposure. Platelets permeabilized in increasing concentrations of OG for 2 min were exposed to 25 μM TRAF for 1 min, and barbed end exposure was determined.

(D) Relationship between the concentration of OG required to permeabilize platelets and detect filament ends. Cells were activated with 25 μM TRAP for 1 min and then permeabilized with 1/10 vol of 10× detergent in PHEM buffer. Data are compared with cells permeabilized in Triton X-100 previously shown to yield maximal detection of filament ends. Each point is the mean ± SD (n = 3).

are now available to promote actin assembly. Increased assembly of actin into filaments was detectable within 15 s after the addition of TRAP. At this timepoint, the number of exposed barbed filament ends increased 3-fold over the resting value. Maximal filament assembly occurred 75 s after TRAP treatment, ~30 s following the highest extent of barbed filament end exposure. TRAP caused rapid changes in phosphoinositide metabolism, resembling previously reported alterations in thrombin-stimulated platelets; PI(4,5)P_2 and PI(4)P decreased transiently but rapidly rose above baseline in conjunction with filament uncapping and net actin assembly.

OG-Permeabilized Platelets Also Expose Barbed Filament Ends When Activated by TRAP

Permeabilization of resting platelets with the detergent n-octyl-β-glucopyranoside (OG) maintains the signal transduction system from the thrombin receptor to actin filament ends. Cells permeabilized with 0.4% OG and then treated with TRAP for 1 min increased the number of free barbed filament ends 3.5-fold (Figure 2A), corresponding to an increase in the number of free barbed ends from the resting level of 90 to 315, ~60% of the sum achieved after TRAP activation of intact cells. Exposure of filament ends was restricted to the barbed ends in the OG-permeabilized cells, since 2 μM cytochalasin B, an agent that predominately binds to the barbed end and inhibits monomer assembly onto this end, abolished all detectable TRAP-stimulated actin assembly. TRAP had no effect on filament ends of platelets permeabilized with Triton X-100 (data not shown).

The retention of TRAP sensitivity in OG-treated platelets was dependent on the detergent concentration. For retention of detectable filament end exposure after ligation of the thrombin receptor with TRAP, 0.4% OG was narrowly optimal. Platelets treated with higher or lower OG concentrations were unresponsive to TRAP (Figures 2C and 3). Filament ends in TRAP-activated cells become detectable between 0.375%–0.5% concentrations of OG, and the maximal sensitivity to TRAP was limited to 0.4%–0.45% OG. These findings imply that TRAP-mediated actin filament uncapping requires both permeable cells and cells that retain sufficient membrane. Observations on the effect of this detergent on the platelet plasma membrane in the electron microscope supported this idea (Figure 3). Small holes, 20–100 nm in diameter, first appear after 0.4% OG treatment (Figure 3B). Increasing the OG concentration further to 0.45% or 0.5% results in extensive disruption of the membrane surface (Figures 3C and 3D).

In both intact and permeabilized cells, doses of TRAP as low as 1.25 μM initiate filament end uncapping, while ≥3.3 μM TRAP maximally exposes barbed filament ends (Figure 2B). TRAP at 3.3 μM has been shown by other investigators to activate platelets optimally as measured by calcium mobilization, secretion, and aggregation (Rasmussen et al., 1993).

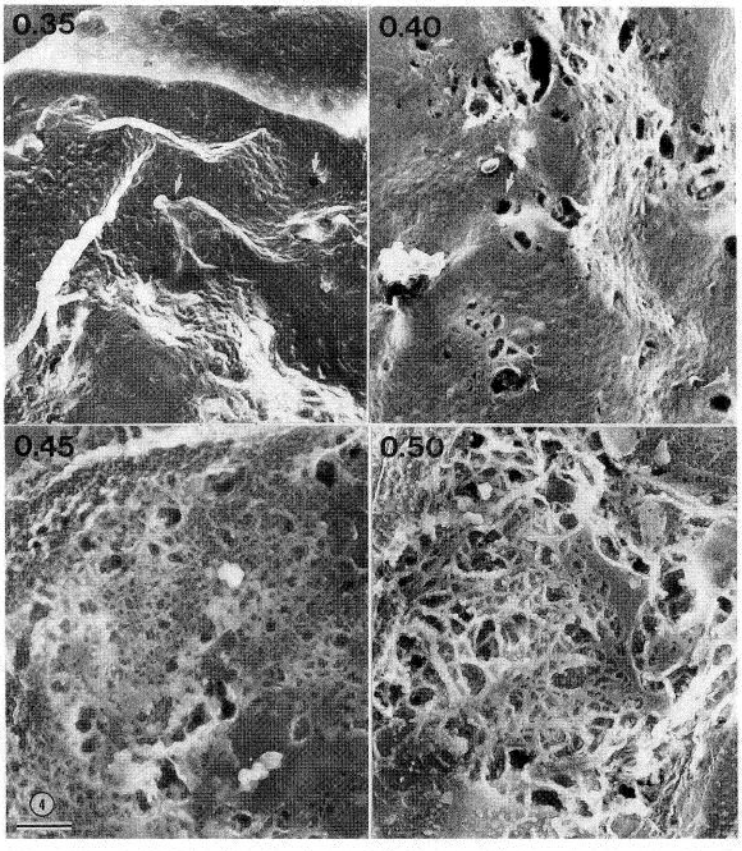

Figure 3. Effect of OG on the Integrity of the Resting Platelet Plasma Membrane

The percent of OG used to treat each platelet sample is indicated in the upper left corner of each micrograph. OG at 0.35% did not permeabilize the plasma membrane. The white arrows show openings of the open canalicular system of the resting platelet. OG at 0.4% produced small holes in the plasma membrane, varying in size ranging from 20 nm to 200 nm in diameter and clustered at various points on the surface, but left large regions of the membrane intact. OG at 0.45% eroded large regions of the plasma membrane, exposing the underlying membrane skeleton. Higher (≥0.5%) concentrations of OG removed most of the membrane and the underlying membrane skeleton. The scale bar represents 200 nm.

Role of Polyphosphoinositides in Barbed Filament End Exposure

The addition of $PI(4,5)P_2$, phosphatidylinositol (3,4)bisphosphate ($PI(3,4)P_2$), or PI(4)P micelles to OG-permeabilized platelets increases the number of free barbed filament ends (Figure 4A). Addition of as little as 20 μM $PI(4,5)P_2$ increased the number of exposed barbed filament ends 2-fold. Maximal barbed end exposure required ~50 μM $PI(4,5)P_2$ and increased the barbed end number 3.0- to 3.5-fold relative to the permeabilized resting cells. Treatment of permeabilized cells with $PI(4,5)P_2$ generated approximately twice as many exposed barbed ends as activation using the TRAP peptide. Treatment of permeabilized cells with PI(4)P also increased the number of barbed ends, although PI(4)P was somewhat less effective than $PI(4,5)P_2$ on a molar basis, and PI(4)P concentrations of >100 μM were required for maximal end exposure. This discrepancy may reflect the larger size of PI(4)P particles compared with micelles of $PI(4,5)P_2$. Phosphatylinositol (3,4,5)trisphosphate ($PI(3,4,5)P_3$) micelles between 10 and 33 μM were as effective as $PI(4,5)P_2$ in generating filament uncapping (Figure 4A). Phosphatidylserine (PS) vesicles had no effect in OG-permeabilized cells, which demonstrates selectivity in the uncapping reaction for the phosphoinositol head group. 1,2-Dioctanolyl-sn-glycerol and 1-oleoyl-2-acetyl-sn-glycerol reportedly promote actin nucleation activity of isolated Dictyostelium membranes (Shariff and Luna, 1992) but had no uncapping activity in permeabilized platelets at concentrations as high as 100 μM. Phosphatidic acid, reported to stimulate actin assembly in intact cells (Ha and Exton, 1993), however, increased the number of free barbed ends 2-fold from 70 ± 40 to 132 ± 63 (mean ± SD, n = 6) at doses between 10 and 33 μM.

Sedimented cytoskeletons partially depleted of soluble compounds also increased their content of detectable free barbed ends in response to $PI(4,5)P_2$ to similar levels as the permeabilized cells, although higher concentrations of $PI(4,5)P_2$ were required to generate maximal filament uncapping (Figure 4B). $PI(4,5)P_2$, in the absence of cytoskeletons, had no effect on the rate at which the pyrene–actin assembled. The increase in the actin assembly rate induced by adding $PI(4,5)P_2$ to cytoskeletons was abolished by the addition of 2 μM cytochalasin B, demonstrating that the effect of the added phospholipid was selective for the barbed ends of filaments.

Stimulation of OG-Permeabilized Platelets with TRAP Leads to the Production of $PI(4,5)P_2$

The major radiolabeled phospholipid detected after TRAP activation of permeabilized cells is $PI(4,5)P_2$ (Figure 5A). $PI(4,5)P_2$ synthesis increased 3-fold 60 s after the addition of TRAP and continued to increase thereafter (Figure 5B).

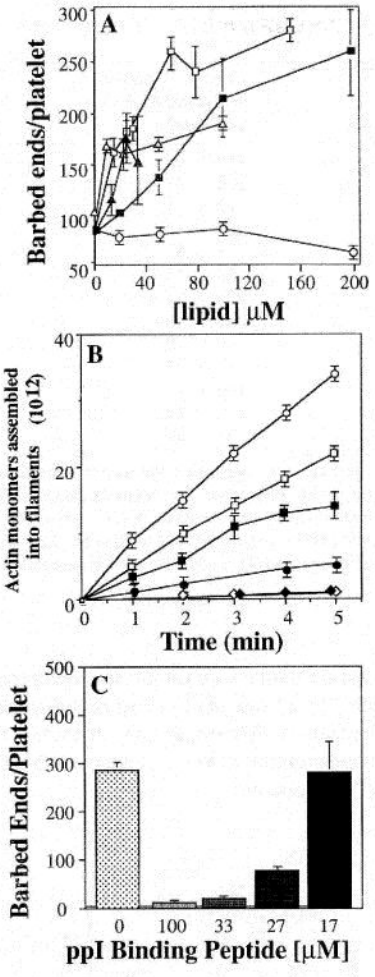

Figure 4. Effect of Lipids or of a Phosphoinositide-Binding Peptide on Filament End Exposure in OG-Permeabilized Resting Platelets or Platelet Cytoskeletons

(A) OG-permeabilized resting platelets were incubated with $PI(4,5)P_2$ (open squares), PI(4)P (closed squares), $PI(3,4)P_2$ (open triangles), $PI(3,4,5)P_3$ (closed triangles), or PS (open circles) for 1 min, and then actin nucleation activity was determined.

(B) Cytoskeletons separated from resting platelet lysates remain responsive to $PI(4,5)P_2$. Cytoskeletons were separated from the soluble proteins in OG-treated resting (squares) or thrombin-activated cells (circles) by centrifugation at 13,000 × g for 5 min in a microcentrifuge at room temperature. The soluble fraction was removed, and pelleted cytoskeletons were resuspended to original volume in 0.1 M KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM EGTA, 0.5 mM 2-mercaptoethanol, 10 mM Tris (pH 7.5), and 100 μl of the suspension was added to each assay. Open circles, cytoskeletons from platelets activated with thrombin for 30 s prior to OG permeabilization; closed circles, cytoskeletons from resting cells; open squares, cytoskeletons from resting cells treated with 150 μM PIP_2 for 1 min prior to their addition to the pyrene–actin assembly assay; closed squares, cytoskeletons from resting cells treated with 75 μM PIP_2 for 1 min prior to their addition to the pyrene–actin assembly assay; open diamonds, actin alone; closed diamonds, actin assembly rate in the presence of 150 μM PIP_2. Each curve represents the mean ± SD of four assays.

(C) Concentration dependence of a 21-mer synthetic peptide corresponding to residues 150–169 of human cytoplasmic gelsolin. Inhibition of TRAP-mediated filament end exposure at 1 min in OG-permeabilized platelets.

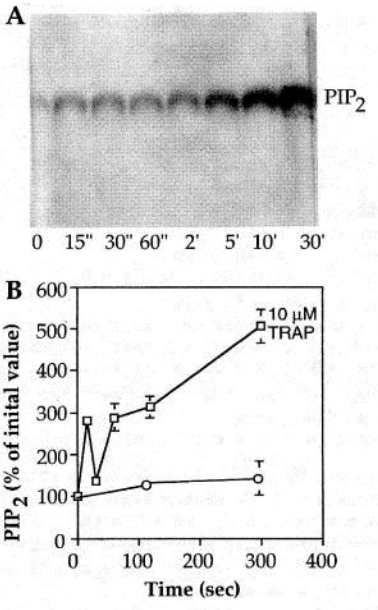

Figure 5. TRAP Treatment of OG-Permeabilized Resting Platelets Results in the Production of PIP_2

(A) Autoradiograph showing the incorporation of [γ-^{32}P]ATP into chloroform–methanol extractable phospholipids.

(B) Quantification of newly synthesized PIP_2 in the platelet lysates.

Peptides That Bind to $PI(4,5)P_2$ Inhibit Filament End Exposure in Response to TRAP

A 21-mer gelsolin-derived ppI-binding peptide at >17 μM inhibited barbed filament end exposure in OG-permeabilized cells by 75% in response to TRAP, while ≥33 μM peptide concentrations completely inhibited TRAP-mediated barbed end exposure (Figure 4C). As shown in Table 1, addition of excess exogenous $PI(4,5)P_2$ to the OG-permeabilized cells overcame the inhibitory effect of the ppI-binding peptide. A shorter peptide from the gelsolin sequence had all the inhibitory effects of the 21-mer, although higher concentrations were required to inhibit filament end exposure in response to TRAP. A number of peptides of similar length and/or charge employed as controls, including a 25-mer highly basic peptide encompassing residues 151–175 of MARCKS, poly-L-lysine, pentalysine, and a 10-mer peptide having a random arrangement of the 10-mer gelsolin ppI-binding sequence, had no detectable effect on the net increase in barbed filament ends induced by TRAP in permeabilized platelets.

GTP Promotes Filament Uncapping While GDP Inhibits Uncapping

Table 1 shows that the addition of either GTP or GTPγS to OG-permeabilized platelets increased the number of free barbed filament ends. Of the two nucleotides, GTPγS was the more effective, generating free barbed filament end numbers comparable to those induced when $PI(4,5)P_2$ was added to the permeabilized cells. GTPγS (at 10 μM) increased barbed end exposure by 4-fold. GTP (at 33 μM)

Table 1. Effect of TRAP, Guanosine Nucleotides, V12Rac GTPase, PI(4,5)P_2, and 21-Mer ppl-Binding Peptide on the Exposure of Barbed Filament Ends in Permeabilized Platelets

Treatment	Change in Number of Barbed Ends per Platelet
10 μM TRAP for 1 min, permeabilize	480 ± 80
Permeabilize, then 10 μM TRAP	200 ± 41
Permeabilize, then 33 μM GTP	130 ± 15
Permeabilize, then 10 μM GTPγS	290 ± 20
Permeabilize, then 100 μM GDPβS	−5 ± 8
Permeabilize, then 10 μM TRAP, 100 μM GDPβS	28 ± 25
Permeabilize, then 33 μM PI(4,5)P_2	272 ± 31
Permeabilize, then 33 μM PI(4,5)P_2 100 μM GDPβS	285 ± 45
Permeabilize, then 10 μM GTPγS, 25 μM 21-mer ppl-binding peptide	25 ± 18
Permeabilize, then 30 μM TRAP, 20 μM ppl-binding peptide	−61 ± 15
Permeabilize, then 30 μM TRAP, 20 μM ppl-binding peptide, 33 μM PI(4,5)P_2	220 ± 41
Permeabilize, 1 μM V12Rac	271 ± 79
Permeabilize, 1 μM V12Rac and 25 μM 21-mer ppl-binding peptide	−11 ± 39

We permeabilized 90 μl of 2 × 10^7 platelets with 10 μl of 4% OG in PHEM buffer for 1 min at 37°C, except for the cells that were activated first and then permeabilized. Permeabilized cells were exposed to the various treatments for 1 min, after which they were diluted to 285 μl with 100 mM KCl, 0.2 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM EGTA, 0.5 mM 2-mercaptoethanol, and 10 mM Tris (pH 7.5). We added 15 μl of a 20 μM pyrene-labeled rabbit skeletal muscle G-actin to start the assay. Barbed ends were calculated as described in the Experimental Procedures. Data are expressed as the mean ± SD for five individual experiments. The number of barbed ends per resting cell was 89 ± 26. The resting end number has been substracted from the activated number.

was less effective and increased the number of free barbed ends by only 2-fold in the OG-permeabilized platelets. GDPβS did not affect the number of ends present in permeabilized resting cells but strongly inhibited uncapping of filament ends induced by TRAP. The addition of PI(4,5)P_2 micelles to lysates treated with GDPβS overcame its inhibition, while the stimulatory effect of GTP was abolished by the addition of the 21-mer PI(4,5)P_2-binding peptide. These results indicate that the effects of GTP analogs are upstream from PI(4,5)P_2 in the signal transduction pathway from TRAP to filament ends.

V12Rac1 Promotes Barbed Filament End Exposure in OG-Permeabilized Platelets

Bacterially expressed consititutively active mutant V12Rac1, but not V14RhoA, stimulated barbed filament end exposure in permeabilized platelets (Figure 6A). V12Rac1, at concentrations ⩾10 nM, freed barbed filament ends to levels comparable to those obtained by adding PI(4,5)P_2 or GTP to the permeabilized cells. Concentrations of ⩾30 nM V12Rac1 resulted in maximal activity. V14RhoA, in contrast, failed to free barbed filament ends even at micromolar concentrations. Identical results were found using recombinant Rac1 and RhoA produced in Sf9 cells using baculovirus and primed with GTP and also with the constitutively active mutant forms of these proteins (data not shown).

V12Rac1 also stimulated the synthesis of PI(4)P and PI(4,5)P_2. V12Rac1 at 3 nM increased PI(4,5)P_2 synthesis by 2.4-fold and barbed end exposure by 3-fold in the OG-permeabilized cells, and concentrations of V12Rac1 >30 nM maximally stimulated both PI(4,5)P_2 synthesis and filament end exposure (Figure 6B). To establish whether the effect of V12Rac1 on filament uncapping was direct or mediated by upstream signaling to phosphoinositide-kinases, experiments were performed using the ppl-binding peptide. The 21-mer ppl-binding peptide at 25 μM completely abolished Rac-dependent filament uncapping (Table 1), indicating that this GTPase functions upstream of PI(4,5)P_2 production.

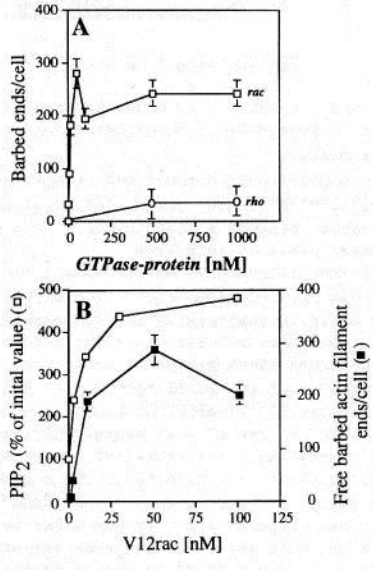

Figure 6. Active Rac but Not Rho Uncaps Barbed Ends and Stimulates PIP_2 Labeling in Permeabilized Platelets

(A) Comparison of the effect of recombinant mutant GTPases V12Rac and V14Rho on filament end exposure in OG-permeabilized platelets.
(B) Mutant recombinant rac lysates stimulates the production of PIP_2 and filament uncapping in OG-permeabilized resting platelets: comparison of dose responses.

Discussion

Permeabilized Platelets That Uncap Actin Filament Barbed Ends in Response to Stimulation

Permeabilization has been a useful approach to advancing understanding of signal transduction mechanisms in cells including platelets (Haslam and Davidson, 1984; King et al., 1991; Kucera and Rittenhouse, 1990). When the signaling involves membrane lipids, as hypothesized for platelet actin remodeling, the challenge is to breach the membrane barrier sufficiently to add intermediates, yet to leave enough membrane intact to participate in messenger activity. We found that this goal could be accomplished with a precise ratio of the detergent OG to platelets that opened 20–100 nm holes in the platelet membrane yet left membrane domains in which thrombin receptors could couple downstream partners, activate phosphoinositide kinases, and synthesize polyphosphoinositides using endogenous substrates spared by the detergent treatment. Platelets permeabilized with 0.4% OG responded to TRAP by uncapping actin filament barbed ends and by concomitantly synthesizing $PI(4,5)P_2$ when supplied with ATP. Higher OG concentrations or another detergent, Triton X-100, rendered the platelets unresponsive.

The extent of barbed end uncapping was greatest in intact platelets stimulated by thrombin receptor ligation, which uncapped twice the number of filaments uncapped in OG-permeabilized and subsequently stimulated cells. One explanation for this difference could be that signaling intermediates were lost during permeabilization. However, one important difference between the two types of preparations is that intact cells are capable of increasing intracellular calcium, whereas the permeabilized cells, being suspended in EGTA-containing buffer solutions, are not. Since the calcium transient of intact cells elicits actin filament severing and increases the number of capped barbed ends available for subsequent uncapping (Hartwig, 1992), the absence of a calcium rise could therefore account for the fewer ends uncapped in permeabilized and subsequently stimulated cells. In support of this explanation is the fact that the number of ends uncapped in these cells approximates the number uncapped in platelets activated under conditions in which intracellular calcium remains in the nanomolar range (Hartwig, 1992) or in cells lacking gelsolin (Witke et al., 1995).

Evidence That Phosphoinositides Are the Terminal Step of Stimulated Actin Assembly in Platelets

First, the D3 and D4 polyphosphoinositides uncap barbed filament ends in OG-permeabilized platelets. Data presented here represent evidence that a phosphoinositide with a phosphate in the 3 as well as the 4 and 5 positions on the inositol ring can uncap actin filament barbed ends. Thrombin (and TRAP) can cause normal actin assembly in platelets treated with inhibitors of phosphoinositide 3-kinase with undetectable D3 phosphoinositide biosynthesis (Kovacsovics et al., 1995), although some types of actin remodeling in platelets and other cells may still result from actin filament uncapping by this class of phospholipids (S. Kung, A. T., T. J. Kovacsovics, and J. H. H., unpublished data). The results also are interesting because previous studies of phosphoinositide effects on gelsolin–actin interactions have shown that these phospholipids are very effective in preventing gelsolin and other capping proteins from binding to actin filaments in vitro, but are inefficient in removing gelsolin and capping protein from actin filament barbed ends except when gelsolin-capped filaments were treated shortly after their formation (Janmey and Stossel, 1989). The ability of phosphoinositides to uncap actin filament barbed ends in permeabilized platelets may be attributable to a different configuration of gelsolin–actin interaction in the platelet compared with in vitro, to capping proteins other than gelsolin, or to as yet unknown factors. The ability to uncap barbed actin filament ends is specific for the three phosphoinositides examined, as several other lipids assayed did not have this effect. On the other hand, phosphatidic acid was about 25%–50% as effective as the polyphosphoinositides in uncapping actin filament barbed ends of OG-permeabilized platelets. Phosphatidic acid stimulates actin assembly in intact cultured fibroblasts (Ha and Exton, 1993), but has no inhibitory effect on the interaction between barbed end capping proteins and actin. Since phosphatidic acid reportedly activates phosphoinositide-synthesizing enzymes (Moritz et al., 1992), its effects on actin assembly could be indirect through the induction of phosphoinositides.

Second, TRAP induces both barbed end actin filament uncapping and phosphoinositide biosynthesis in intact and OG-permeabilized platelets. The time course of phosphoinositide synthesis is rapid and could therefore account for local phospholipid accumulation capable of uncapping actin filament barbed ends. The total phosphoinositide concentrations in resting platelets, if in the form of clusters, are high enough to uncap actin filaments in permeabilized platelets. Therefore, not all of this lipid is accessible to capped barbed ends, and the newly generated phosphoinositides are required for uncapping.

Third, peptides derived from gelsolin that specifically bind $PI(4,5)P_2$ with high affinity (Janmey et al., 1992), but not several control peptides that lack such binding, are potent inhibitors of stimulus-induced actin filament barbed end uncapping in OG-permeabilized platelets.

The GTPase Rac Leads to Phosphoinositide Synthesis-Mediated Barbed End Uncapping of Actin Filaments

Recent work has elucidated how different stimuli can promote particular types of actin assembly within cells (Hall, 1993, 1994). One key ingredient of this specification is the species of GTPase recruited by the stimulus. For example, acting through the GTPase Rho, lysophosphatidic acid in serum builds adhesion plaques and actin filament–rich stress fiber bundles in certain cultured cells (Jalink et al., 1994; Ridley and Hall, 1992). Working through the GTPase Rac, growth factors direct actin remodeling in pleat-like protrusions, the ruffles, of such cells (Ridley et al., 1992).

Yet another GTPase, CDC42, appears to program the actin-based assembly that results in protrusion of hair-like filopodia and is an upstream regulator of Rac (Nobes and Hall, 1995). Given these important clues and the additional findings that Rho reportedly stimulates PI(4)P 5-kinase in vitro (Chong et al., 1994) and that nonhydrolyzable GTP analogs stimulate cellular phosphoinositide generation (Stephens et al., 1993), we were logically directed to examine whether addition or perturbation of these GTPases would uncap barbed ends of actin filaments in OG-permeabilized platelets.

Our findings indicate that particular GTPases stimulate phosphoinositide kinase activities that uncap actin filaments in specific locations (Figure 7). First, GTP and GTPγS alone promoted barbed filament end uncapping in permeabilized platelets; GDPβS, predicted to inhibit the transducing activity of GTPases, not only failed to induce uncapping but inhibited TRAP-activated uncapping. Similar stimulatory or inhibitory effects on actin assembly by GTP and GDPβS, respectively, have been observed in permeabilized neutrophils (Redmond et al., 1994). We showed that PI(4,5)P_2 micelles, however, produced barbed end actin filament uncapping in OG-permeabilized platelets exposed to GDPβS. These results would be expected if GTP hydrolysis precedes phosphoinositide synthesis in the pathway to actin filament uncapping. Second, we found that constitutively active mutant V12Rac, but not active V14Rho, elicited barbed end uncapping in parallel with activation of PI(4,5)P_2 biosynthesis in OG-permeabilized platelets. Third, induction of actin filament barbed end uncapping in OG-permeabilized platelets by GTP and GTPγS, and by constitutively active Rac were inhibited by the same concentrations of PI(4,5)P_2-binding peptide that prevented TRAP-induced barbed end uncapping.

In summary, OG-permeabilized platelets retain stimulus-responsive signaling pathways that lead to an actin filament barbed end uncapping reaction. Evidence presented here points to phosphoinositide-mediated uncapping of actin filament ends as the last step in this pathway. GTPase activity, driven by Rac, works upstream to stimulate phosphoinositide biosynthesis. This regulatory design could program the precise spatial generation of the phosphoinositides near actin filament barbed ends. In platelets, particular GTPases could program the different types of actin assembly leading to lamellae or filopodia. This system should lend itself to further elucidation of steps involved in what will undoubtedly be a complex and fascinating control panel regulating the platelet shape change. The lessons learned should also be applicable to other kinds of cells.

Experimental Procedures

Preparation of Resting Platelets

Human blood from healthy volunteers, drawn into 1/10 vol of Aster–Jandl anticoagulant, was centrifuged at 110 × g for 10 min. The platelet-rich plasma was gel filtered at room temperature through a Sepharose 2B column, equilibrated, and eluted with a solution containing 145 mM NaCl, 10 mM HEPES, 10 mM glucose, 0.5 mM Na_2HPO_4, 5 mM KCl, 2 mM $MgCl_2$, and 0.3% bovine serum albumin (pH 7.4) (platelet buffer). Cells were incubated for 30 min at 37°C to insure a resting state.

Permeabilization of Platelets

Resting platelets, ~2 × 10^8/ml, were lysed using 0.1 vol of OG (final concentrations of 0.1%–0.7%), 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 20 μM phallacidin, and 42 nM leupeptin, 10 mM benzamidine, and 0.123 mM aprotinin to inhibit proteases (Hartwig, 1992). To determine the minimal (rest) and maximal (active) number of filament ends exposed in cells, resting platelets were permeabilized by the addition of 0.1 vol of above buffer containing 0.75% Triton instead of OG before or 60 s after addition of 10 μM TRAP. In some experiments, the cytoskeletal fraction was separated from the total lysate by centrifugation at 12,300 × g for 5 min in a Sorvall Microspin centrifuge. The cytoskeletal pellet that resulted from this centrifugation was resuspended at its original volume in the above buffer lacking added detergent.

Activation of Platelets

Platelets in suspension, detergent-permeabilized platelets from resting cells, or the cytoskeletal portion of permeabilized platelets were activated by the addition of thrombin receptor–activating peptides, either the 14-residue (TRAP) or the 6-residue (TRAF) forms (Bachem, King of Prussia, PA), for 60 s. When intact cells were used, activation was terminated by permeabilizing the cells as detailed below.

Fluorescence Measurement of Filament End Status

The effect of cell lysates on the rate and extent of pyrene-labeled actin was determined as described previously (Hartwig, 1992). Resting or TRAP-activated cells (90 μl) were permeabilized with 10 μl of 4% OG, or 100 μl of permeabilized resting cells were exposed to TRAP or

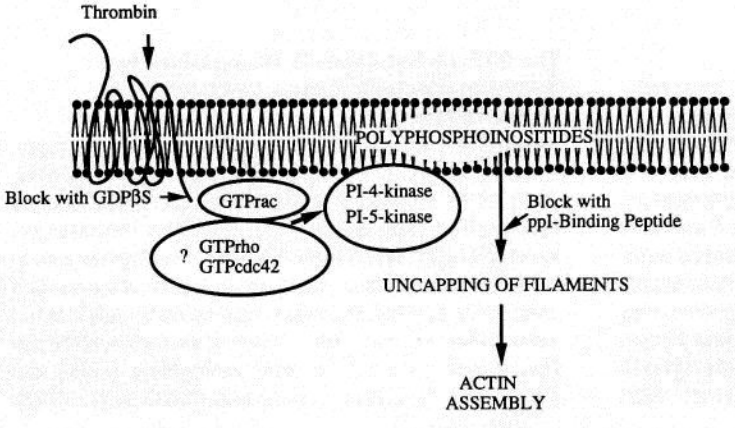

Figure 7. Summary Scheme of the Pathway from the Thrombin Receptor to Actin Filament Ends in Platelets

The thrombin receptor couples to phosphoinositide kinases through the small G protein Rac. The ppI-binding peptides block the exposure of newly synthesized phosphoinositides.

TRAF for 60 s and then added to 185 μl of 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM EGTA, 0.5 mM dithiothreitol, and 10 mM Tris (pH 7.0). The polymerization rate assay started by the addition of monomeric pyrene-labeled rabbit skeletal muscle actin to a final concentration of 1 μM. The relative contribution of exposed barbed or pointed filament ends in detergent-permeabilized cells was determined by adding 2 μM cytochalasin B to the pyrene–actin assembly assay. Pyrene–actin assembly onto actin filament ends is completely inhibited at the barbed end by 2 μM cytochalasin B in this assay (Hartwig, 1992). The barbed end assembly rate is calculated by subtracting the assembly rate in the presence of cytochalasin B from the assembly rate in the absence of cytochalasin B. Activity not inhibited by cytochalasin B in this assay derives from the pointed end of filaments. Barbed end numbers were calculated from the number of actin monomers adding to the barbed end per second (derived from the change in fluorescence per second) divided by the actin monomer addition rate at the barbed end (10 subunits/s) at the added 1 μM actin concentrations and the number of cells per assay (1.8×10^7 cells).

Measurement of Actin Filament Assembly

A rhodamine–phalloidin-based flow cytometry assay was used to quantitate the actin filament content of resting and activated platelets (Howard and Oresajo, 1985). The fluorescence of bound rhodamine–phalloidin is proportional to the total amount of F-actin. Platelets under resting or activating conditions were fixed by the addition of an equal volume of 4% paraformaldehyde in platelet buffer without BSA for 30 min at 37°C. Fixed platelets were made permeable with a final concentration of 0.1% Triton X-100 and labeled with 10 μM rhodamine–phalloidin for 30 min at room temperature. The samples were immediately read in a Beckton-Dickinson FACScan flow cytometer. The samples were gated for platelets based on their forward- and side-scatter profile. The results are expressed as percent F-actin.

Phospholipids

Phosphatidylserine (dipalmitoyl-L-α-phosphatidylserine) (PS), PI(4)P, $PI(4,5)P_2$, phosphatidic acid (PA), and 1,2-dioctanolyl-sn-glycerol (diC8) were obtained from Sigma (St. Louis, MO). $PI(3,4)P_2$ was purchased from Matreya, Incorporated (Pleasant Gap, PA). $PI(3,4,5)P_3$ was a gift from Drs. D.-M. Gou and C.-S. Chen (College of Pharmacy, University of Rhode Island, Kingston, RI) (Gou and Chen, 1994). PA, PS, PI(4)P, $PI(4,5)P_2$, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ were dissolved by sonication in water as described by Janmey and Stossel (1989). diC8 was prepared and added to platelet lysates as described by Shariff and Luna (1992).

Polyphosphoinositide-Binding Peptides

Two peptides based on a ppI-binding site in gelsolin (Janmey et al., 1992) were synthesized and HPLC purified: a 10-mer peptide encoding gelsolin residues 160–169 (QRLFQVKGRR) (a gift of R. Vegners, Latvian Institute of Organic Synthesis, Riga, Latvia) and a 21-mer peptide containing residues 150–169 of cytoplasmic gelsolin to which a cysteine residue was added at the amino terminus (CKHVVPNEVVVQRLFQVKGRR). Control peptides include a 25-mer peptide based on the sequence of MARCKS, residues 151–175 (acetyl-KKKKKRFSFKKSFKLSGFSFKKNKK), that was a gift of A. Aderem, Rockefeller University (New York, New York) and a random peptide having the same residues as the gelsolin 10-mer (FRVKLKQGQR). All peptides were dissolved in water.

Small GTP-Binding Proteins

Recombinant bacterial V12Rac1 and V14RhoA were prepared by Dr. T. Azuma using cDNAs provided by Dr. A. Hall (University College London, London, England) by the method of Ridley et al. (Ridley and Hall, 1992; Ridley et al., 1992). Recombinant processed GTPase proteins were purified from Sf9 insect cell using a baculovirus expression system (Heyworth et al., 1993) as described by Chong et al. (1994). All GTPase proteins were charged with GTP using a 10-fold excess of GTP to GTPase protein in 20 mM Tris, 1 mM EDTA, 100 mM NaCl, and 1 mM DTT (pH 7.5) for 15 min at 30°C. The GTP-loading reaction was stopped by adding 5 mM $MgCl_2$.

Assay of Phospholipid Mass and Production

Gel-filtered platelets (10^9/ml) were incubated with 2 mCi/ml [^{32}P]orthophosphoric acid for 1 hr at 37°C. Unbound ^{32}P was separated from the cells by gel filtration in the presence of 1 μM postaglandin E1. Resting and activated lipids were analyzed as described in Kovacsovics et al. (1995).

Resting platelets at concentrations from 2×10^8–3×10^8/ml were permeabilized for 1 min at 37°C using a final OG concentration of 0.4%. Permeabilized cells were then treated with the TRAP peptide or recombinant V12Rac1 protein in the presence of 50–100 μCi/ml of [γ-^{32}P]ATP, added along with the activating agent. At different times, the reaction was stopped by removing 1 ml of the lysate solution. Lysates were processed by two procedures for TLC analysis.

Procedure 1

Solution (1 ml) was added to 3.8 ml of a 20:40:1:2 solution of chloroform:methanol:12 N HCl:0.1 M EDTA and vigorously mixed using a vortex for 20 s (Auger et al., 1989a, 1989b). To this solution, 1.25 ml each of ice-cold chloroform and distilled water were added. The solution was mixed using a vortex, and its phases were separated by centrifugation at 1300 × g for 10 min at 4°C. The upper phase was discarded, and the lower phase was washed a second time with 2.5 ml of 3:48:7 solution of chloroform:methanol:0.6 N HCl. The phases were again separated by centrifugation, the upper phase was discarded, and the organic phase was dried under nitrogen gas, washed with 1 ml of chloroform, and dried. Dried material was resuspended in 200 μl of chloroform containing 0.5 mM butylated hydroxytoluene. Each sample (40 μl) was applied to one lane of a 20 × 20 cm Silica gel 150A TLC plate (Whatman International Limited, Maidstone, England) that had been pregrooved (19 channels). The TLC plate was coated with 1% potassium oxalate and dried at 110°C for a minimum of 30 min prior to sample application. Phospholipids were separated by chromatography in 60:47:11.3:2 of chloroform:methanol:water:8.56 M NH_4OH. The location of PIP, PIP_2, and PIP_3 was determined using a known standard solution. Plates were stained by placing them in a saturated iodine tank. ^{32}P incorporated into phospholipids was revealed by autoradiography.

Procedure 2

Lysate (1 ml) was added to 300 μl of 3 M HCl followed by 2 ml of 50:50 chloroform:methanol (Whitman et al., 1987). The organic and water phases were separated by centrifugation at 12,300 × g for 30 s, the water phase was discarded, and the chloroform:methanol phases were dried under nitrogen. Samples were resuspended in chloroform containing 0.5 mM butylated hydroxyltoluene, and phospholipids were separated by TLC using a solvent of 65:35 of 1-propanol:2 M acetic acid. Plates were stained by placing them in a saturated iodine tank. ^{32}P incorporated into phospholipids was revealed by autoradiography. The density of radiolabeled phospholipid bands was quantified by digitizing the image followed by pixel counts (Molecular Dynamics).

Electron Microscopy

Resting platelets suspended in platelet buffer were treated with final concentrations of the detergent OG from 0.2%–0.5% for 2 min and attached to the surface of polylysine-coated glass coverslips by centrifugation at 280 × g for 5 min. The cells were fixed with 1% glutaraldehyde in PHEM buffer for 10 min, washed extensively into distilled water, rapidly frozen, freeze dried at −90°C, and coated with 1.4 nm of platinum with rotation and 2.5 nm of carbon without rotation (Cressington CFE-50 apparatus, Watford, England). Replicas were separated from the coverslips in 25% hydrofluoric acid, cleaned, and picked up on carbon–formvar-coated copper grids. Specimens were photographed at 100 kV in a JEOL-1200 EX electron microscope.

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