Phosphatidylinositol 4,5-Bisphosphate Regulates Activation-Induced Platelet Microparticle Formation†

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ABSTRACT: While the role of the cytoskeleton in microparticle formation is well-described, the role of membrane phospholipids in regulating this process is poorly defined. PIP_2 binds many cytoskeletal proteins and may oppose microparticle formation through associations with these proteins. To determine whether PIP_2 effects microparticle formation, PIP_2 was incorporated into platelet membranes prior to activation-induced microparticle formation. Incorporation of PIP_2 into platelet membranes inhibited activation-induced microparticle formation by ≥90%. Inhibition was dose-dependent with an IC_{50} of 12−18 μM. A permeabilized platelet system was next used to assess the effect of modulation of endogenous PIP_2 levels on microparticle formation. Infusion of type IIβ PIP kinase into permeabilized platelets inhibited microparticle formation by 75 ± 8%. In contrast, incubation of permeabilized platelets with PI-specific phospholipase C augmented microparticle formation by greater than 3-fold. Evaluation of PIP kinases following platelet activation demonstrated that they were lost from platelets in a calpain-dependent manner during microparticle formation. Purified μ-calpain cleaved recombinant type IIβ PIP kinase and inhibited its ability to phosphorylate PI(5)P. In permeabilized platelets, incubation of purified μ-calpain reduced PIP_2 levels, while exposure to calpeptin increased PIP_2 levels. Calpain has previously been implicated in platelet microparticle formation. These studies show that calpain may help limit PIP_2 formation following platelet activation and that PIP_2 content is an important determinant of platelet microparticle formation.

Upon activation with a potent agonist, platelets shed small vesicles that are termed microparticles from their surface (*1*). Platelet microparticles were first recognized for their procoagulant activity in vitro (*2*) and are thought to contribute to normal hemostasis (*1*). They can be formed from platelets following incubation with pharmacologic agonists (*1*), complement binding proteins (*3*), or high shear (*4*) or as the result of platelet storage (*5*), yet the underlying molecular events that culminate in the vesiculation of a membrane from platelets are not well-understood. Several mechanisms, including activation-induced proteolysis of the membrane skeleton (*6*), protein tyrosine dephosphorylation (*7*), protein phosphorylation (*8*), and calmodulin activation (*8*), have been implicated in the process. One essential component of microparticle generation is elevation of intracellular Ca^{2+} levels (*9*). For example, in platelets exposed to complement proteins C5b-9, millimolar concentrations of Ca^{2+} are used to elicit microparticle formation (*9*, *10*). Ca^{2+} may elicit microparticle formation via interactions with anionic phospholipids, Ca^{2+}-binding proteins, or both. Calpain is perhaps the best-characterized Ca^{2+}-binding protein shown to participate in microparticle formation. Inhibition of calpain using small molecules can prevent platelet microparticle formation induced by thrombin and collagen, Ca^{2+} ionophore, or dibucaine, which activates calpain (*6*, *11*−*13*). Calpain cleavage of proteins of the membrane skeleton correlates with vesiculation of platelet membranes and represents one compelling mechanism of inducing vesiculation (*12*). These calpain substrates include cytoskeletal−structural proteins such as cortactin, talin, filamin (*14*, *15*), and the cytoplasmic tail of integrin β_3 (*16*). The fact that microparticles can be formed in the presence of inhibitors of calpain by incubation with complement C5b-9 and Ca^{2+}, however, demonstrates that calpain-independent mechanisms of microparticle formation exist (*9*). Thus, activation of calpain is not the most distal event in platelet microparticle formation, and/or multiple pathways leading to microparticle formation exist.

In other cell types, detachment of the plasma membrane from the cortical actin cytoskeleton is considered central to processes such as membrane shedding (*17*), blebbing (*18*, *19*), and vesiculation (*20*). There is some evidence that specific phospholipids in the plasma membrane contribute to the association of the membrane with the underlying cortical cytoskeleton and/or membrane skeleton. Interference with the association of these phospholipids with the cytoskeletal components results in vesiculation. Phosphatidylinositol 4,5-bisphosphate (PIP_2)[1] is an example of a lipid component that has been invoked in maintaining the membrane−cytoskeleton association. Loss of PIP_2 from erythrocyte membranes is associated with microvesicle formation

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(*21*, *22*). More recently, PIP_2 in membranes has been demonstrated to increase membrane−cytoskeleton adhesion energy (*23*, *24*). This adhesion force is mediated at least in part by the binding of proteins of the membrane skeleton to PIP_2. For example, PIP_2 binds the PH domain of β-spectrin with a K_d of ~40 μM (*25*), suggesting that PIP_2 can mediate membrane−cytoskeleton adhesion via low-affinity interactions with high-copy number proteins. In addition, PIP_2 interacts with other actin-binding proteins found in platelets, such as α-actinin, ezrin, radixin, and moesin, with considerably higher affinity (*26*, *27*). Thus, PIP_2 is considered an important determinant of membrane−cytoskeleton adhesion.

Since PIP_2 contributes to membrane−cytoskeleton association, we sought to determine whether it regulates platelet microparticle formation. We found that PIP_2 is a potent inhibitor of platelet microparticle formation. Studies in permeabilized platelets demonstrated that microparticle formation was inhibited by elevation of endogenous PIP_2 levels and augmented by depression of PIP_2 levels. Calpain has previously been shown to cleave enzymes that mediate phosphatidylinositol metabolism in platelets (*28*, *29*). Given our observation that PIP_2 controls platelet microparticle formation and the established role of calpain in platelet microparticle formation, we sought to determine whether calpain influences PIP_2 metabolism in platelets. These studies demonstrated that PIPKs are cleaved by calpain following platelet activation. In addition, calpain controls activation-dependent PIP_2 levels following platelet activation. These observations indicate that PIP_2 is an important determinant of microparticle formation and that calpain proteolysis of proteins such as PIPKs regulates PIP_2 levels.

MATERIALS AND METHODS

Chemicals and Reagents. All buffer constituents, solvents, Coomassie Blue, MgATP, SL-O, PI, PIP, PIP_2, PI-specific PLC, brain extract, A23187, Fura-2, and $CaCl_2$ were purchased from Sigma (St. Louis, MO). PC, PS, and PE were obtained from Avanti Polar Lipids (Alabaster, AL). α-Thrombin was purchased from Hematologic Technologies, Inc. (Essex Junction, VT), and collagen was purchased from BioData Corp. (Horsham, PA). Calpain I purified from porcine erythrocytes, calpeptin (Z-Leu-Nle-CHO), calpastatin, α-toxin, and bisindolylmaleimide I were purchased from Calbiochem (San Diego, CA). [^{3}H]PIP_2 and [^{32}P]orthophosphoric acid were obtained from New England Nuclear (Boston, MA). PI(5)P was purchased from Echelon Research Laboratories (Salt Lake City, UT). Calcein-AM and R18 were purchased from Molecular Probes, Inc. (Eugene, OR). FITC-labeled annexin V was obtained from BD Biosciences (San Jose, CA). Silica gel G TLC plates were obtained from Whatman Ltd. (Kent, England). Goat polyclonal affinity-purified antipeptide antibodies to type II PIPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody was raised against a peptide consisting of the 19 amino-terminal amino acids of type IIα PIPK. This antibody recognized a single band of 53 kDa in platelet lysates and recognized a recombinant type II PIPK−GST fusion protein in immunoblot analysis (*30*). The antibody directed against type I PIPK was kindly provided by C. Carpenter (*31*). GST-tagged type IIβ PIPK was generously provided by L. Rameh. All solutions were prepared using water purified by reverse-phase osmosis on a Millipore Milli-Q Purification Water System.

Platelet Preparation. Blood from healthy donors who had not ingested aspirin in the two weeks prior to donation was collected by venipuncture into 0.4% sodium citrate. Citrate-anticoagulated blood was centrifuged at 200*g* for 20 min to prepare platelet-rich plasma. Platelets were then purified from platelet-rich plasma by gel filtration using a Sepharose 2B column equilibrated in 25 mM PIPES, 137 mM NaCl, 4 mM KCl, and 0.1% glucose (pH 6.4) for experiments using intact platelets or 25 mM PIPES, 2 mM EGTA, 137 mM KCl, 4 mM NaCl, and 0.1% glucose (pH 6.4) for experiments using permeabilized platelets (*30*, *32*, *33*). Platelet samples were adjusted to pH 7.5 prior to being used in functional assays. Final gel-filtered platelet concentrations were approximately 2×10^8 platelets/mL.

Permeabilization of Platelets. Platelets were permeabilized using reduced SL-O or α-toxin using the concentrations indicated in the figure legends (*33*). The ability of each batch of toxin to permeabilize platelets was tested by analyzing each for incorporation of FITC−dextran sulfates by flow cytometry as described previously (*32*).

Analysis of Platelet Microparticle Formation from Intact Platelets. To distinguish between platelet microparticles and phospholipid micelles by flow cytometry, calcein-AM-labeled platelets were used to generate calcein-labeled platelet microparticles. Gel-filtered platelets were incubated in 1.25 μg/mL calcein-AM for 15 min. Calcein-AM-labeled platelets were then incubated with either thrombin and collagen or A23187 and 1 mM Ca^{2+}. Microparticles generated from calcein-AM-labeled platelets were evaluated using fluorescence detected in the FL-1 channel versus right-angle side light scatter. Fluorescent microparticles derived from calcein-AM-labeled platelets were readily distinguished from unlabeled phospholipid micelles. Microparticles were quantified as a percentage of total events [microparticles/(microparticles + platelets) × 100] (*8*, *34*). Calcein-AM labeling itself had no effect on microparticle particle formation under the conditions used in these experiments. Flow cytometry was performed using a Becton-Dickinson FACSCalibur flow cytometer. Fluorescent channels were set at logarithmic gain. Ten thousand particles were acquired for each sample. A 530/30 band-pass filter was used for FL1 fluorescence to detect calcein. Data were analyzed using CellQuest software on a MacIntosh PowerPC.

Analysis of Platelet Microparticle Formation from Permeabilized Platelets. To evaluate the generation of platelet microparticles from permeabilized platelets, gel-filtered platelets were incubated with 1000 units/mL SL-O in PIPES/EGTA buffer (pH 7.5) and the indicated concentration of Ca^{2+} for 20 min. Platelet microparticles generated from SL-O-permeabilized platelets bound annexin V to the same extent as platelet microparticles generated by exposure of intact platelets to 5 μM A23187 and 1 mM Ca^{2+}. To enable

[1] Abbreviations: FITC, fluorescein isothiocyanate; PIPES, 1,4-piperazinediethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKC, protein kinase C; SD, standard deviation; SL-O, streptolysin-O; type I PIPK, type I phosphatidylinositol 4-phosphate 5-kinase; type II PIPK, phosphatidylinositol 5-phosphate 4-kinase; R18, octadecyl rhodamine B; TLC, thin-layer chromatography.

the distinction between platelet microparticles and phospholipid micelles in these experiments, microparticles generated from permeabilized, calcein-AM-labeled platelets were evaluated using fluorescence detected in the FL-1 channel versus right-angle side light scatter as described for intact platelets. Microparticles were quantified as a percentage of total events.

Analysis of PIP_2 Transfer. The transfer of PIP_2 into membranes was analyzed as previously described (*35*). Briefly, [^{3}H]PIP_2-labeled micelles were prepared at 1 mM with a specific activity of 10 mCi/mmol of PIP_2. Micelles were incubated with gel-filtered platelets for 30 min at the concentrations indicated in the figure legends. [^{3}H]PIP_2-labeled micelles were subsequently separated from platelets by centrifugation at 2000*g* for 10 min. Radioactivity associated with the washed platelet pellet and with the unincorporated [^{3}H]PIP_2-labeled micelles was determined using a Tri-carb 2100TR Liquid Scintillation Analyzer (Packard, Albertville, MN). The amount of PIP_2 transferred to platelets was calculated from the platelet number, the specific activity of [^{3}H]PIP_2, and the radioactivity detected in the platelet pellet.

R18 Membrane Fusion Assay. Fusion of PIP_2 micelles with platelet membranes was monitored using dequenching of R18 as previously described (*36*, *37*). Briefly, R18-labeled PIP_2 micelles containing 1 mM PIP_2 and 10 μM R18 were prepared. R18 that was not incorporated into micelles was removed by centrifugation at 150000*g* and 4 °C for 1 h. Platelets (50 μL) were mixed with R18-labeled PIP_2 micelles to a final concentration of 50 μM PIP_2 at 37 °C. Samples in which R18-labeled PIP_2 micelles were mixed with buffer alone served as controls. R18 fluorescence was measured with excitation at 560 nm and emission at 590 nm at 30 min using a GeminiXS spectrofluorometer (Molecular Devices, Sunnyvale, CA). The fusion reaction was stopped by addition of Triton X-100 at a final concentration of 0.1% (v/v). Fusion was measured by fluorescence dequenching (%Fd) which was calculated as follows: $\%Fd = (F - F_o)/(F_{max} - F_o) \times 100$. F is the fluorescence intensity following incubation of micelles with platelets at the time point indicated in the figure legend. F_o is the fluorescence intensity of micelles mixed with buffer alone. F_{max} is the fluorescence intensity of the following disruption of membranes by Triton X-100, which yields maximum dequenching.

Analysis of Platelet Phosphoinositides. Analysis of PIP_2 levels in platelets was performed as previously described (*30*). Briefly, gel-filtered platelets (350 μL/sample) were incubated in the presence of 2 mCi/mL [^{32}P]orthophosphoric acid at 37 °C for 4 h. Unbound ^{32}P was separated from platelets by centrifugation. ^{32}P-labeled platelets were then permeabilized in the presence of Ca^{2+} and buffer or recombinant proteins as indicated. Following a 20 min incubation, platelets were solubilized in a 20:40:1 solution of chloroform, methanol, and 12 N HCl. Phospholipids were extracted and separated by TLC in a 47:60:3 solution of methanol, chloroform, and ammonium hydroxide. The locations of PIP and PIP_2 were determined by comparison to known standards applied to each lane. The radioactivity on the plates was detected and quantified using a Typhoon 9400 Molecular Imager (Amersham, Piscataway, NJ).

Kinase Assays. Kinase assays were performed as previously described (*38*). Briefly, recombinant type IIβ PIPK bound to Sepharose beads was assayed in 50 μL reaction mixtures containing 23 mM HEPES (pH 7.0), 100 mM NaCl, 30 μM $MgCl_2$, 4 μg of PI(5)P, 36 μg of brain extract, and 150 μM [γ-^{32}P]ATP (10 μCi/assay). Reactions were stopped after 10 min by adding 25 μL of 5 M HCl and then 160 μL of chloroform and methanol (1:1). Lipids were separated by TLC using 1-propanol and 2 M acetic acid (65:35, v/v), and PIP_2 was quantified as described above.

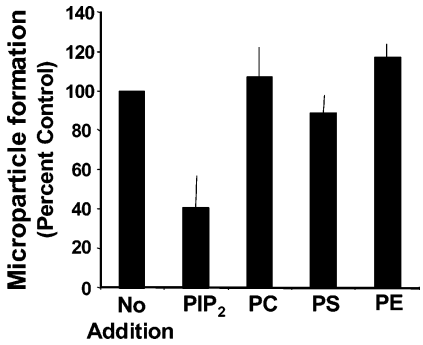

FIGURE 1: Effect of phospholipids on activation-induced platelet microparticle formation. Calcein-AM-labeled platelets were incubated with buffer alone (No Addition) or 12.5 μM phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylcholine (PC), phosphatidylserine (PS), or phosphatidylethanolamine (PE). Following a 20 min incubation, microparticle formation was induced using 5 μM A23187 in the presence of 1 mM Ca^{2+}. Microparticle formation was subsequently analyzed by flow cytometry. Data are expressed as the percent of microparticles formed compared to controls that were incubated in the absence of phospholipid. Error bars represent the SD of four independent experiments.

Gel Electrophoresis. Gel-filtered platelets ($1-2 \times 10^7$ per milliliter) were exposed to buffer, 100 μM SFLLRN, or 10 μM Ca^{2+} and 500 units/ml SL-O as indicated in the figure legends. Following a 10 min incubation, samples were pelleted and solubilized in sample buffer (62.5 mM Tris-HCl, 2% SDS, 0.5% β-mercaptoethanol, 14% glycerol, and 0.01% bromophenol blue) at 95 °C for 5 min. Platelet proteins were then separated by SDS−PAGE on 14% gels. Immunoblotting was performed using the indicated anti-PIPK antibodies and visualized using enhanced chemiluminescence. For silver stain analysis, gels were fixed in a 40:10:50 ethanol/acetic acid/ddH_2O solution. Proteins were detected using the SilverQuest Staining Kit according to the protocol of the supplier (Invitrogen, Carlsbad, CA).

RESULTS

PIP_2 Inhibits Platelet Microparticle Formation. While the role of the cytoskeleton in platelet microparticle formation has previously been evaluated (*6*, *12*, *39*, *40*), the role of membrane constituents in this process is poorly defined. To investigate the effect of lipid composition on platelet microparticle formation, we incubated platelets with various phospholipids prior to stimulation with calcium ionophore A23187. Platelets and platelet microparticles were analyzed by flow cytometry as previously described (*1*, *7*, *8*). However, calcein-AM-labeled platelets were used to generate microparticles in these studies to allow a distinction to be made between platelet microparticles and phospholipid micelles by flow cytometry. In initial studies, platelets were incubated in the presence of 12.5 μM PIP_2, PC, PS, or PE. At this phospholipid concentration, incubation with PIP_2 resulted in a 59 ± 9% reduction in the level of microparticle formation (Figure 1). Incubation with PC, PS, or PE had little effect

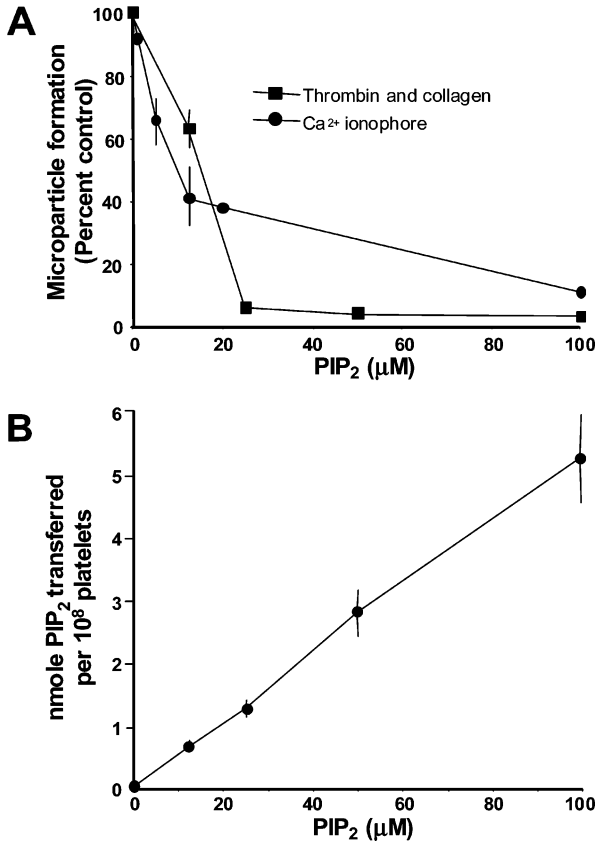

FIGURE 2: Dose-dependent inhibition of platelet microparticle formation by PIP_2. (A) Calcein-AM-labeled platelets were incubated with the indicated concentrations of PIP_2. Following a 20 min incubation, microparticle formation was induced using either 1 unit/mL thrombin and 50 μg/mL collagen (■) or 5 μM A23187 (●) in the presence of 1 mM Ca^{2+}. Microparticle formation was subsequently analyzed by flow cytometry. Data are expressed as the percent of microparticles formed compared to controls that were incubated in the absence of PIP_2. Error bars represent the SD of three independent experiments. (B) Gel-filtered platelets were incubated with the indicated concentrations of $[^3H]PIP_2$-labeled micelles. Following this incubation, platelets were washed by centrifugation. The amount of $[^3H]PIP_2$ that remained associated with the platelet was quantified using a scintillation counter. The quantity of PIP_2 transferred to platelets was calculated as described in Materials and Methods. Error bars represent the SD of six independent experiments.

on microparticle formation. These results demonstrate that PIP_2 preferentially inhibits agonist-induced microparticle formation compared with other phospholipids.

To further evaluate the effect of PIP_2 levels on platelet microparticle formation, platelets were incubated with increasing concentrations of PIP_2 micelles and subsequently stimulated with either thrombin and collagen or the Ca^{2+} ionophore A23187. Exposure of platelets to PIP_2 micelles inhibited microparticle formation induced by either agonist by $\geq$90% (Figure 2A). Inhibition was dose-dependent with an IC_{50} of 10−16 μM. Microparticle formation is dependent on elevation of the intracellular Ca^{2+} concentration (*41*, *42*), and PIP_2 is able to bind and chelate Ca^{2+}. We therefore assessed the effect of incubation with PIP_2 micelles on A23187-induced Ca^{2+} levels using Fura-2. This analysis demonstrated that intracellular Ca^{2+} levels rose to 3.5 ± 1.1 μM in the absence of PIP_2 exposure and to 3.2 ± 0.8 μM following exposure to 100 μM PIP_2. This result suggests that PIP_2 does not inhibit microparticle formation by chelating Ca^{2+}. To determine the quantity of PIP_2 that was transferred upon incubation of platelets with PIP_2 micelles, we used $[^3H]$-PIP_2-labeled micelles. The transfer of PIP_2 occurred in a dose-dependent manner. Incubation of platelets with 12.5 μM PIP_2 micelles resulted in the transfer of 0.7 ± 0.1 nmol of $PIP_2/10^8$ platelets (Figure 2B), representing 21.7 ± 3.2% of the available PIP_2 in micelles. To determine whether PIP_2-labeled micelles simply bound to platelets or actually fused with platelet membranes, an R18 dequenching fusion assay was performed. Relief of R18 self-quenching in lipid bilayers is used to monitor membrane fusion (*36*, *37*). For these studies, R18 was incorporated into PIP_2 micelles at 1% mole fraction and subsequently incubated with platelets. Fusion occurred in a time-dependent manner such that 21 ± 3.4% of micellar PIP_2 (i.e., the vast majority of PIP_2 micelles that interact with platelets) fused with platelets following a 30 min incubation. These results demonstrate that PIP_2 micelles fuse with platelet membranes prior to inhibition of microparticle formation.

Endogenous PIP_2 in Platelet Microparticle Formation. The observation that exogenously added PIP_2 micelles inhibit platelet microparticle formation led us to evaluate the role of endogenous PIP_2 in platelet micorparticle formation. For this purpose, we developed an SL-O-permeabilized platelet model of microparticle formation. Permeabilization of platelets with SL-O permits the entry of molecules as large as 260 kDa into the platelet cytosol (*32*). This permeabilization method enables introduction of proteins with specific activities such as antibodies and enzymes into platelet cytosol to establish the importance of particular processes in microparticle formation. For these studies, microparticles were formed by incubation of platelets with SL-O in the presence of 1 mM free Ca^{2+}. Microparticles formed in this system exhibited forward and side scatter characteristics similar to those of microparticles generated from intact platelets exposed to A23187. The level of binding of annexin V to platelet microparticles generated from SL-O-permeabilized platelets exposed to Ca^{2+} was 115 ± 2% of the level of binding of annexin V to microparticles generated from intact platelets. To enable the distinction of platelet microparticles from phospholipid micelles used in these studies, microparticles were generated from calcein-AM-labeled platelets. There was some loss of calcein from platelets following permeabilization. However, sufficient calcein was incorporated into microparticles generated from calcein-AM-labeled permeabilized platelets to enable detection of fluorescent microparticles (Figure 3A). The degree of microparticle formation in this permeabilized platelet system was approximately 35% of that induced by A23187 and approximately 130% of that obtained upon incubation of platelets with thrombin and collagen (Figure 3B). Exposure of platelets to 1 mM Ca^{2+} in the absence of SL-O or to 1000 units/mL SL-O alone resulted in only negligible microparticle formation. Incubation of permeabilized platelets with PIP_2 micelles inhibited Ca^{2+}-induced microparticle formation from SL-O-permeabilized platelets (Figure 3C). The sensitivity to PIP_2 of Ca^{2+}-dependent microparticle formation from SL-O-permeabilized platelets was similar to that observed in agonist-stimulated intact platelets.

We next used the permeabilized platelet model to determine the effect of increasing endogenous PIP_2 levels on microparticle formation. For these studies, permeabilized

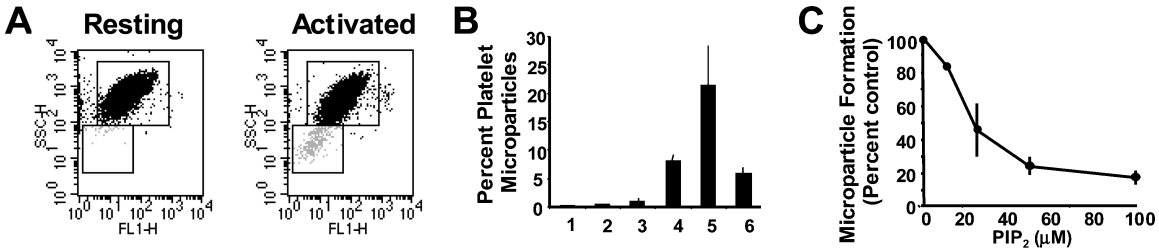

FIGURE 3: Microparticle formation from SLO-permeabilized platelets. (A) Gel-filtered, calcein-AM-labeled platelets were exposed to 1000 units/mL SL-O in the absence (Resting) or presence (Activated) of 1 mM Ca^{2+}. Following a 20 min incubation, samples were assayed by flow cytometry. (B) Calcein-AM-labeled platelets were incubated in the presence of (1) buffer alone, (2) 1 mM Ca^{2+}, (3) 1000 units/mL SL-O, (4) 1 mM Ca^{2+} followed by 1000 units/mL SL-O, (5) 5 μM A23187 in the presence of 1 mM Ca^{2+}, or (6) 1 unit/mL thrombin and 50 μg/mL collagen. Following a 20 min incubation, microparticle formation was quantified by flow cytometry. Data represent microparticles as a percent of total events (microparticles and platelets). Error bars represent the SD of four to six independent experiments. (C) Calcein-AM-labeled platelets were incubated with the indicated concentration of PIP_2 and subsequently permeabilized with 1000 units/mL SL-O in the presence of 1 mM Ca^{2+}. Following a 20 min incubation, platelet microparticle formation was analyzed by flow cytometry. Data are expressed as the percent of microparticles formed compared to controls that were incubated in the absence of PIP_2. Error bars represent the SD of three independent experiments.

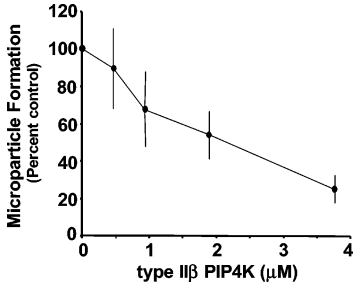

FIGURE 4: Elevation of endogenous PIP_2 levels inhibits platelet microparticle formation. Platelets were permeabilized with 1000 units/mL SL-O in the presence of 2 mM MgATP, 1 mM Ca^{2+}, and the indicated concentration of type IIβ PIPK. Following a 20 min incubation, platelet microparticle formation was analyzed by flow cytometry. Data are expressed as the percent of microparticles formed compared to controls that were incubated in the absence of type IIβ PIPK. Error bars represent the SD of four independent experiments.

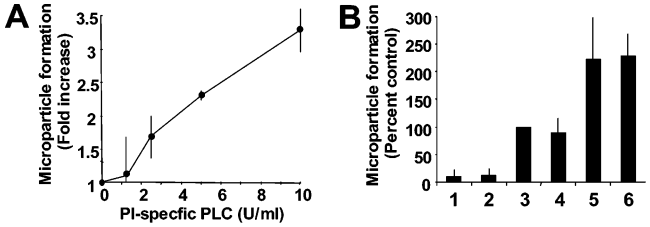

FIGURE 5: Hydrolysis of endogenous PIP_2 augments platelet microparticle formation. (A) Platelets were permeabilized with 1000 units/mL SL-O in the presence of 750 μM Ca^{2+} and the indicated concentrations of PI-specific PLC. Following a 20 min incubation, platelet microparticle formation was analyzed by flow cytometry. Data are represented as the fold increase over microparticle particle formation from Ca^{2+}-stimulated samples incubated in the absence of PI-specific PLC. Error bars represent the SD of four independent experiments. (B) Platelets were permeabilized in the presence of (1) buffer alone, (2) 7.5 units/mL PI-specific PLC, (3) 750 μM Ca^{2+}, (4) 750 μM Ca^{2+} and bisindolylmaleimide I, (5) 750 μM Ca^{2+} and PI-specific PLC, or (6) 750 μM Ca^{2+}, bisindolylmaleimide I, and PI-specific PLC. Following a 20 min incubation, platelet microparticle formation was analyzed by flow cytometry. Data are expressed as the percent of microparticles formed compared to Ca^{2+}-stimulated controls that were incubated in the absence of PI-specific PLC. Error bars represent the SD of three to six independent experiments.

platelets were incubated with type IIβ PIPK. Incubation of permeabilized platelets with 3.75 μg/mL type IIβ PIPK resulted in a 50 $\pm$ 4% increase in PIP_2 levels as measured in [^{32}P]orthophosphoric acid-labeled platelets. Incubation of permeabilized platelets with increasing concentrations of type IIβ PIPK inhibited Ca^{2+}-induced microparticle formation in a dose-dependent manner (Figure 4). Incubation of unpermeabilized platelets with recombinant type IIβ PIPK had no effect on platelet microparticle formation (data not shown). These data indicate that elevation of endogenous PIP_2 levels inhibits platelet microparticle formation.

We next sought to determine whether depression of endogenous PIP_2 levels augments platelet microparticle formation. PI-specific PLC was used to hydrolyze endogenous PIP_2. Following incubation of SL-O-permeabilized platelets with 5 units/mL PI-specific PLC, PIP_2 levels were 24.3 $\pm$ 11.6% of PIP_2 levels in untreated platelets (*30*). Microparticle formation was then assessed in platelets permeabilized with SL-O in the presence or absence of the indicated concentrations of PI-specific PLC and Ca^{2+}. Under these conditions, PI-specific PLC augmented platelet microparticle formation in a dose-dependent manner. The extent of microparticle formation was increased 2.3 $\pm$ 0.1-fold at 5 units/mL PI-specific PLC (Figure 5A). The use of PI-specific PLC in permeabilized platelets, however, is complicated by the fact that the byproducts of PI-specific PLC activity, diacylglycerol and inositol 1,4,5-trisphosphate, are capable of activating signaling pathways that could influence microparticle formation (*43*). Diacylglycerol acts primarily via activation of protein kinase C (*44*). To evaluate the possibility that incubation of permeabilized platelets with PI-specific PLC generates diacylglycerol that can augment microparticle formation by activating protein kinase C, experiments were performed in the presence of bisindolylmaleimide I. Bisindolylmaleimide I is a selective inhibitor of protein kinase C that inhibited PMA-induced platelet granule secretion (data not shown). This inhibitor failed to prevent PI-specific PLC-dependent augmentation of microparticle formation, suggesting that activation of protein kinase C was not responsible for augmenting the activity of PI-specific PLC (Figure 5B). PI-specific PLC had no effect on platelet microparticle formation when incubated with permeabilized platelets in the absence of Ca^{2+} (Figure 5B). In addition, PI-specific PLC failed to augment Ca^{2+} ionophore-induced microparticle formation in intact platelets (data not shown). These results indicate that reduction of endogenous PIP_2 levels augments microparticle formation.

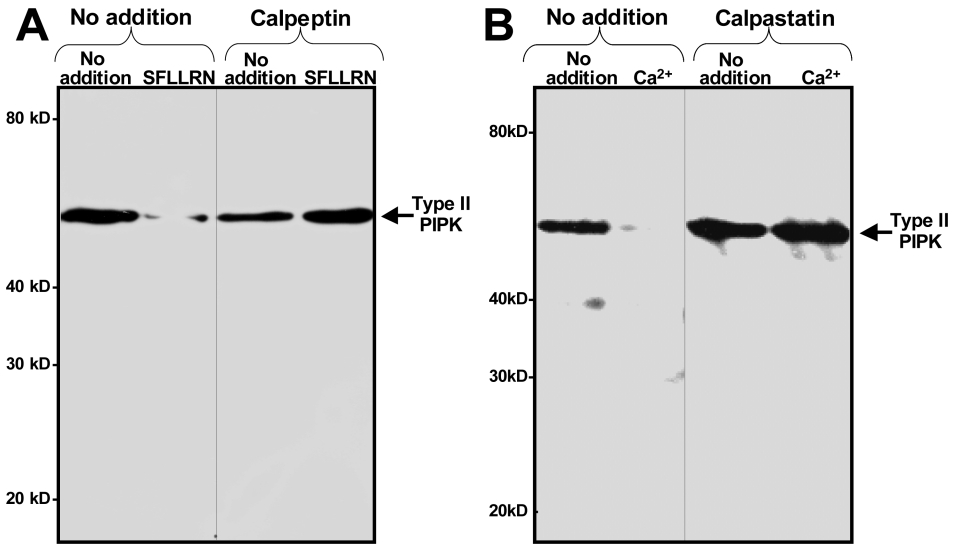

FIGURE 6: Calpain cleaves type II PIPK following activation of platelets. (A) Gel-filtered platelets were incubated in the presence (Calpeptin) or absence (No addition) of 50 μM calpeptin. Platelets were subsequently exposed to buffer (No addition) or SFLLRN (SFLLRN). Following a 15 min incubation, platelets were pelleted. Proteins from platelet pellets were assayed for type II PIPK by immunoblotting. (B) Platelets were incubated in the presence (Calpastatin) or absence (No addition) of 50 μg/mL calpastatin. Platelets were then permeabilized with 500 units/mL SL-O in the presence (Ca^{2+}) or absence (Buffer) of Ca^{2+}. Following a 10 min incubation, platelets were solubilized in sample buffer and platelet proteins were assayed for type II PIPK by immunoblotting.

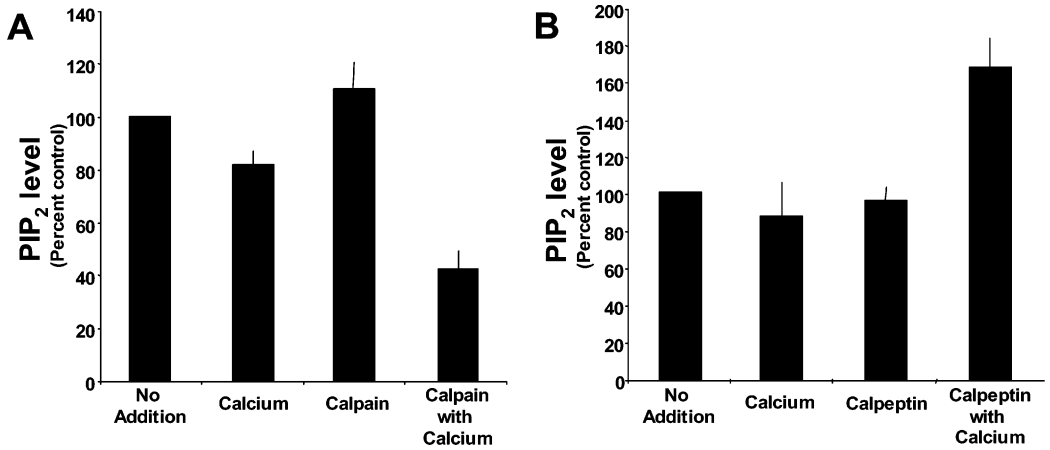

FIGURE 7: Calpain regulates PIP_2 levels in permeabilized platelets. (A) ^{32}P-labeled gel-filtered platelets were permeabilized with 500 units/mL SL-O and incubated in the presence of (1) buffer alone, (2) Ca^{2+}, (3) 2 units/mL μ-calpain alone, or (4) 2 units/mL μ-calpain and Ca^{2+}. Platelets were subsequently analyzed for PIP_2 by TLC as described in Materials and Methods. Error bars represent the standard error of the mean of six independent experiments. (B) ^{32}P-labeled platelets were permeabilized with 125 units/mL α-toxin and incubated in the presence of (1) buffer alone, (2) Ca^{2+}, (3) 50 μM calpeptin alone, or (4) 50 μM calpeptin and Ca^{2+}. Platelets were then analyzed for PIP_2 by TLC. Data are expressed as the percent PIP_2 level compared with samples exposed to buffer alone. Error bars represent the standard error of the mean of six independent experiments.

Calpain Cleaves PIPKs. PIP_2 is generated primarily by phosphorylation of PIP by PIPKs. While evaluating the fate of type II PIPK during microparticle formation, we found that it is lost from platelets following stimulation by SFLLRN (Figure 6) or the Ca^{2+} ionophore (data not shown). In addition, type II PIPK was lost from permeabilized platelets exposed to Ca^{2+} (Figure 6). These observations raised the possibility that type II PIPK is cleaved by a Ca^{2+}-sensitive protease following platelet activation. Calpain is a Ca^{2+}-dependent protease that is abundant in platelet cytosol (*45*). We next used inhibitors of calpain to determine whether calpain mediated the activation-dependent loss of type II PIPK from platelets. Loss of type II PIPK following stimulation with SFLLRN was inhibited by calpeptin, a cell-permeable calpain inhibitor (Figure 6A). Similarly, loss of type II PIPK from Ca^{2+}-treated, SL-O-permeabilized platelets was inhibited by calpastatin, a protein that specifically inhibits calpain (Figure 6B). Incubation of purified μ-calpain with recombinant type IIβ PIPK resulted in cleavage of the PIPK and loss of its kinase activity (data not shown). PEST sequences are amino acid sequences that confer susceptibility to calpain (*46*, *47*). Evaluation of the amino acid sequence of type IIβ PIPK demonstrated a PEST sequence localized to an unstructured region between the helix α7 and strand β11 of the seven-stranded antiparallel β-sheet (*48*). Evaluation of the amino acid sequences of the α, β, and γ isoforms of type I and II PIP kinases demonstrated that all these proteins contain PEST sequences (Table 1). As predicted by these PEST sequences, type I PIPKs were also cleaved following platelet activation in a calpain-dependent manner (data not shown). These observations demonstrate that calpain cleaves and inactivates PIPKs.

Calpain Influences PIP_2 Levels in Platelets. Since calpain cleaves several enzymes involved in PIP_2 metabolism,

Table 1: PEST Sequences with PIPK Isoforms

type I PIPK	amino acids	PEST score
type Iα	324−338	5.03
	496−539	7.97
type Iβ	77−89	8.98
type Iγ	505−538	7.24
	591−645	12.36
type II PIPK	**amino acids**	**PEST score**
type IIα	308−342	2.23
type IIβ	301−335	1.25
type IIγ	128−140	14.46
	292−303	10.92

including PIPKs, we sought to test the possibility that calpain can influence PIP_2 levels in platelet membranes. In these experiments, platelets were permeabilized in the presence or absence of purified μ-calpain, exposed to either buffer or Ca^{2+}, and subsequently analyzed for PIP_2 by TLC. Introduction of purified calpain into permeabilized platelets in the presence of Ca^{2+} decreased PIP_2 levels by approximately 55% ($P < 0.005$) (Figure 7A). To determine whether endogenous calpain controls PIP_2 levels upon platelet activation, permeabilized platelets were incubated in the presence or absence of calpeptin prior to exposure to Ca^{2+}. Incubation of permeabilized platelets with calpeptin enhanced PIP_2 levels following exposure to Ca^{2+} by 70% ($P < 0.04$) (Figure 7B). These observations support the supposition that calpain controls activation-dependent PIP_2 levels.

PIP_2 and Calpain-Mediated Microparticle Formation. In addition to cleaving proteins involved in phosphatidylinositol metabolism, calpain cleaves multiple substrates within the platelet cytoskeleton (*12*). Calpain-induced proteolysis of the membrane skeleton has been demonstrated to participate in microparticle formation (*12*). We therefore used calpain to determine whether inhibition of microparticle formation by PIP_2 occurred at a step proximal to proteolysis of the membrane skeleton in inhibiting microparticle formation. If PIP_2 was acting on a proximal signaling event, then it would be unable to inhibit calpain-induced platelet microparticle formation. For these experiments, we incubated SL-O-permeabilized platelets with 2 units/mL purified calpain to degrade the platelet cytoskeleton and subsequently assessed microparticle formation. Purified calpain augmented Ca^{2+}-induced microparticle formation by approximately 1.8-fold (Figure 8A). Degradation of actin-binding proteins was confirmed by monitoring cleavage of talin and filamin (Figure 8B). Exposure of permeabilized platelets to PIP_2 micelles inhibited microparticle formation in the presence or absence of calpain equally well. Thus, PIP_2 can overcome the influence of calpain upon microparticle formation. PIP_2 did not inhibit calpain-meditated cleavage of talin and filamin (Figure 8B), consistent with previous observations that PIP_2 actually augments calpain activity (*49*). These results demonstrate that the effects of calpain proteolysis on microparticle formation can be reversed by increasing PIP_2 levels in the platelet membrane. Therefore, PIP_2 is not acting by inhibiting proximal signaling events. Rather, PIP_2 appears to act at a distal event. This observation suggests that degradation of the membrane skeleton is not sufficient for microparticle formation in the presence of a noncompliant membrane, and thus, membrane composition is an important determinant of microparticle formation.

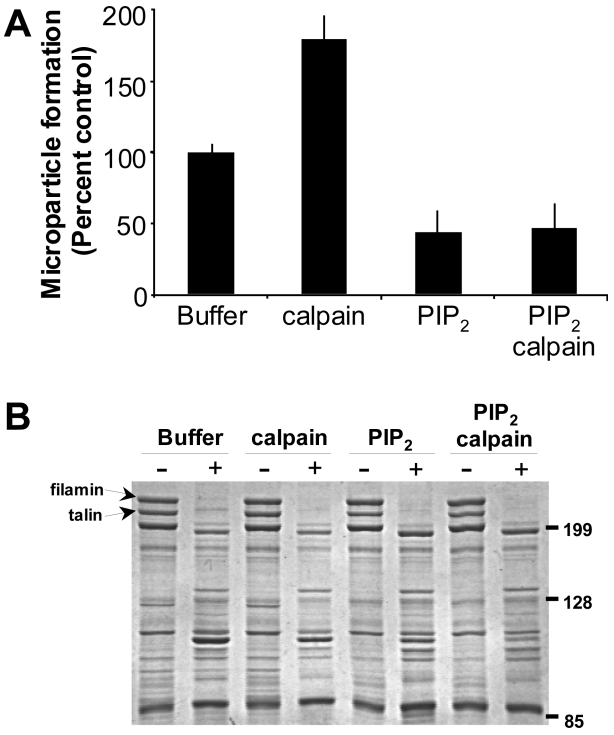

FIGURE 8: PIP_2 inhibits calpain-augmented stimulation of platelet microparticle formation. (A) Calcein-AM-labeled platelets were exposed to buffer, 2 units/mL purified calpain, 50 μg/mL PIP_2, or 2 units/mL calpain and 50 μg/mL PIP_2 as indicated. Platelets were subsequently permeabilized with 1000 units/mL SL-O in the presence of 750 μM Ca^{2+}. Following a 15 min incubation, samples were analyzed for microparticles using flow cytometry. Data are expressed as the percent of microparticles formed compared to Ca^{2+}-stimulated controls that were incubated with buffer alone. Error bars represent the SD of four independent experiments. (B) Platelets were exposed to buffer, 2 units/mL purified calpain, 50 μg/mL PIP_2, or 2 units/mL calpain and 50 μg/mL PIP_2 as indicated. Platelets were subsequently permeabilized with 1000 units/mL SL-O in the presence (+) or absence (−) of 750 μM Ca^{2+}. Platelet proteins were analyzed by SDS−PAGE and visualized by staining with Coomassie blue. Arrows designate filamin and talin.

We next sought to determine whether loss of PIP_2 could facilitate microparticle formation in the absence of calpain-induced cleavage of the membrane skeleton. Under the conditions of our assay, microparticle formation following permeabilization of platelets in the presence of Ca^{2+} was significantly inhibited in the presence of calpeptin (Figure 9A). Calpeptin also inhibited Ca^{2+}-induced proteolysis of filamin and talin in this system (Figure 9B). To determine whether loss of PIP_2 can facilitate microparticle formation in the absence of calpain activity, platelets were incubated with calpeptin to inhibit calpain activity and PI-specific PLC to lower endogenous PIP_2 levels. PI-specific PLC augmented microparticle formation from platelets treated with Ca^{2+} alone as well as from platelets treated with Ca^{2+} and calpeptin. Incubation of permeabilized platelets with PI-specific PLC enhanced microparticle formation from calpeptin-exposed platelets to levels similar to those of platelets treated with Ca^{2+} alone. Thus, PI-specific PLC cleaves PIP_2 and facilitates microparticle formation even in the absence of calpain-mediated degradation of the membrane skeleton.

DISCUSSION

Our results demonstrate that PIP_2 in platelet membranes opposes the formation of microparticles. This conclusion is

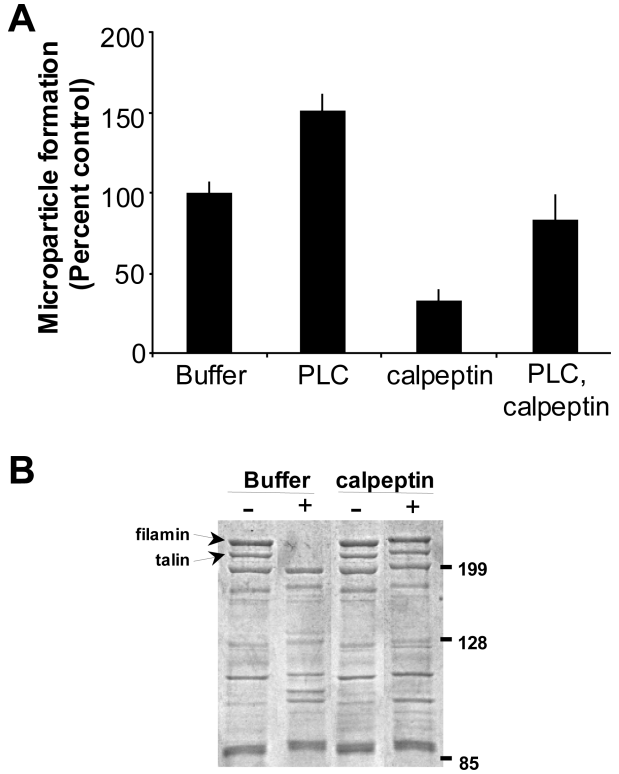

FIGURE 9: Loss of PIP_2 results in microparticle formation in the absence of calpain activity. (A) Gel-filtered platelets were exposed to buffer, 7.5 units/mL PI-specific PLC, 50 μM calpeptin, or 7.5 units/mL PI-specific PLC and 50 μM calpeptin as indicated and permeabilized in the presence of 500 μM free Ca^{2+}. Following a 15 min incubation, samples were analyzed for microparticles using flow cytometry. Data are expressed as the percent of microparticles formed compared to Ca^{2+}-stimulated controls that were incubated with buffer alone. Error bars represent the SD of four independent experiments. (B) Platelets were exposed to buffer or 50 μM calpeptin and subsequently permeabilized with 1000 units/mL SL-O in the presence (+) or absence (−) of 500 μM Ca^{2+}. Platelet proteins were analyzed by SDS−PAGE and visualized by staining with Coomassie blue. Arrows designate filamin and talin.

based on the observations that exogenously added PIP_2 micelles inhibit platelet microparticle formation, that the infusion of a PIPK into platelets inhibits microparticle formation, and that cleavage of endogenous PIP_2 by PI-specific PLC augments microparticle formation. The multiple roles of PIP_2 in platelet signal transduction and membrane dynamics render it difficult to unambiguously define the mechanism by which PIP_2 inhibits platelet microparticle formation. Potential mechanisms of PIP_2-mediated inhibition of microparticle formation include Ca^{2+} chelation and disruption of agonist-induced signaling. However, incubation with PIP_2 did not alter the elevation of the intracellular Ca^{2+} concentration as analyzed using Fura-2. Similarly, at concentrations that inhibit microparticle formation, PIP_2 failed to inhibit α-granule secretion induced by thrombin and collagen or A23187 (data not shown). On the basis of these observations, we propose that PIP_2 regulates microparticle formation at a distal element in the signaling pathway.

Several effects of PIP_2 on distal events required for microparticle formation may explain its ability to inhibit this process. PIP_2 may prevent microparticle formation by binding proteins of the platelet cytoskeleton. For example, PIP_2 has been demonstrated to contribute to membrane−cytoskeleton adhesion energy, and its presumed mechanism is binding to cytoskeletal proteins (*23*, *24*, *50*). These interactions include low-affinity binding to membrane skeleton components of high copy number such as spectrin and higher-affinity binding to less abundant actin binding proteins found in platelets, such as α-actinin, ezrin, radixin, and moesin (*26*, *27*). The ability of PIP_2 to inhibit microparticle formation following calpain-induced cleavage of talin and filamin (Figure 8) suggests that PIP_2 does not act by binding these membrane skeleton components. However, PIP_2 could bind to membrane skeleton components that are resistant to calpain, such as moesin (*51*), or proteins that are incompletely degraded. PIP_2 levels also affect physical properties of platelet membranes, including local charge density, local membrane curvature, lateral tension, and packing properties. PIP_2-mediated changes in membrane properties may oppose membrane fusion events required for microparticle formation. Future studies will evaluate whether PIP_2 inhibits microparticle formation by associating with proteins of the underlying membrane skeleton, preventing fusion of membranes required for microparticle formation, or some combination of these two mechanisms.

PIPKs are essential components of PIP_2 generation in platelets (*52*, *53*). Several observations support the supposition that PIPKs are cleaved by calpain during platelet microparticle formation. PIPKs are lost from platelets in a Ca^{2+}-dependent manner following platelet activation. Inhibitors of calpain such as calpeptin and calpastatin inhibit Ca^{2+}-dependent loss of PIPKs (Figure 6). The primary amino acid sequence of type I and II PIPKs includes PEST sequences that confer susceptibility to intracellular proteases such as calpain (Table 1) (*46*). The susceptibility of PIPKs to calpain in platelets and the observation that PIPKs contain PEST sequences raise the possibility that PIPKs in other cells may be proteolyzed by calpain.

Platelet PIP_2 levels are regulated by multiple mechanisms, including PIPKs, lipases, phosphatases, and PI 3-kinases. Calpain exerts control over PIP_2 levels in platelets by cleaving several proteins that are either directly or indirectly involved in PIP_2 metabolism. Examples of this level of regulation include calpain-mediated cleavage of $PLC_{\beta 3}$, which may augment the activity of this lipase (*29*), and cleavage of PIPK isoforms, which inhibits the activity of these phospholipid kinases (data not shown). The differential contribution of these targets in mediating the effect of calpain on PIP_2 remains to be determined, yet the supposition that calpain controls PIP_2 levels is supported by the observations that incubation of permeabilized platelets with purified calpain reduces PIP_2 levels and that incubation with calpeptin results in the elevation of PIP_2 levels (Figure 7). These results raise the possibility that calpain facilitates the vesiculation of the membrane from the platelet surface in part by depressing activation-induced PIP_2 levels. This function of calpain would synergize with its previously described role in proteolyzing proteins of the membrane skeleton (*6*, *12*).

Our studies demonstrate that cytoskeletal proteolysis and membrane composition are both important components of platelet microparticle formation. Infusion of recombinant calpain into permeabilized platelets augments microparticle formation (Figure 8), yet PIP_2 can inhibit calpain-induced augmentation of microparticle formation without interfering with the ability of calpain to proteolyze proteins of the membrane skeleton (Figure 8). This observation suggests that

calpain-mediated proteolysis of cytoskeletal components does not result in microparticle formation unless the membrane is permissive. We have also found that hydrolysis of endogenous PIP_2 by PI-specific PLC can augment platelet microparticle formation even in the presence of calpeptin, a potent calpain inhibitor (Figure 9). These results demonstrate that microparticle formation can occur in the absence of degradation of the membrane skeleton. Thus, cleavage of the membrane skeleton is not the sole determinant of microparticle formation. Membrane phospholipids contribute to the regulation of platelet microparticle formation, and in particular, PIP_2 opposes this process.

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