Aggregation-Dependent Signaling in Human Platelets Is Sensitive to Protein Serine/Threonine Phosphatase Inhibitors†

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ABSTRACT: When platelets are stimulated by the addition of thrombin, a series of temporally linked signaling events are initiated. Some of the early events are needed to engage the integrin glycoprotein (GP) IIb-IIIa in a high-affinity state. This in turn leads to aggregation, which initiates a wave of events distinct from those triggered by thrombin. Platelet responses are sensitive to protein serine/threonine phosphatase inhibitors, but which events are dependent on protein phosphatase activity is not known. In the present studies, the effect of the phosphatase inhibitor calyculin A on aggregation-induced signaling was examined. The addition of 0.2 unit/mL thrombin caused aggregation-dependent redistribution of cytoskeletal proteins (actin binding protein, talin, vinculin, and α-actinin), glycoproteins (GPIIb-IIIa, PECAM), and signaling molecules (PI3-kinase, pp60$^{c\text{-}src}$) to the cytoskeletal fraction of platelets. Addition of 1−2 μM calyculin A blocked the ability of 0.2 unit/mL thrombin to induce aggregation and the association of these molecules with the cytoskeleton. Aggregation (60−80% of control) was restored if 1 unit/mL thrombin was added, but there was no corresponding redistribution of actin binding protein, talin, vinculin, α-actinin, GPIIb-IIIa, PECAM, PI3-kinase, and pp60$^{c\text{-}src}$ to the cytoskeleton. Treatment of platelets with calyculin A resulted in an increase in the phosphorylation state of a membrane skeletal protein of 50 kDa. These data strongly suggest that platelet aggregation is dissociable from aggregation-induced signaling, which is dependent on type 1 and 2A phosphatase activities.

A link between protein phosphorylation reactions and platelet responses was first demonstrated by Lyons et al. (1975). Their work, and that of Haslam and co-workers (Haslam et al., 1979), established that protein phosphorylation reactions are part of (and modulate) a cascade of events that control platelet reactivity. Since these early experiments, it has been well demonstrated that platelet agonists such as thrombin initiate a multitude of signaling cascade events, including protein kinase C activation, Ca^{2+} mobilization, and protein tyrosine phosphorylations, and that seryl, threonyl, and tyrosyl phosphorylation events are integral reactions of the activation pathway (Nishizuka, 1984; Ferrell & Martin, 1988; Golden & Brugge, 1989). It is unclear whether distinct phosphorylation events are spatially or temporally linked. In support of a temporal relationship, Ferrell and Martin (1989) demonstrated that waves of protein tyrosine phosphorylations occur in response to thrombin: Some of the phosphorylations were dependent on platelet aggregation, emphasizing that propagation of a signal depends on events that occur subsequent to those signaling events directly initiated by thrombin's interaction with platelets.

More recent studies have shown that direct occupancy of platelet adhesion molecules and the consequent aggregation induce signaling events. For example, under stirring conditions, occupancy of the major platelet integrin glycoprotein (GP)[1] IIb-IIIa with fibrinogen causes rapid changes in tyrosine phosphorylations of specific proteins (Ferrell & Martin, 1989; Golden et al., 1990) and the association of adhesion molecules (GP IIb-IIIa, PECAM) (Newman et al., 1992; Fox et al., 1993), cytoskeletal proteins (vinculin, talin, α-actinin) (Kouns et al., 1991; Fox et al., 1993), and the signaling molecules (PI3-kinase, pp60$^{c\text{-}src}$, pp62$^{c\text{-}yes}$, pp72^{syk}, rap1b) (Fischer et al., 1990; Grondin et al., 1991; Fox et al., 1993; Horvath et al., 1992; Oda et al., 1992; Zhang et al., 1992; Yanagi et al., 1994) with the cytoskeletal fraction. GPIIb-IIIa exists in a low-affinity state on circulating platelets. Thus, some form of signaling is a prerequisite for priming GPIIb-IIIa before these aggregation-induced changes are observed [reviewed in Shattil et al. (1994)], further supporting a temporal relationship between signaling pathways. As expected on the basis of this model, inhibiting the sequence of events upstream of GPIIb-IIIa activation [by either elevating intracellular cAMP levels (Watson et al., 1984) or inhibiting protein phosphatases (Lerea, 1992)] attenuates aggregation.

Types 1 (PP1) and 2A (PP2A) protein serine/threonine phosphatases have been identified in platelets (Lerea, 1991; Erdodi et al., 1992), and their activities appear to be critical for platelet functions (Karaki et al., 1989; Lerea, 1991; Murphy & Westwick, 1994). The addition of the serine/threonine phosphatase inhibitors calyculin A or okadaic acid

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[1] Abbreviations: DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; GPIIb-IIIa, glycoprotein IIb-IIIa; GP1b, glycoprotein 1b; HRP, horseradish peroxidase; PI, phosphatidylinositol; PECAM, platelet endothelial cell adhesion molecule 1; PRP, platelet-rich plasma; PGE_1, prostaglandin E_1; PP1, protein phosphatase type 1; PP2A, protein phosphatase 2A; RGD, arginine-glycine-aspartic acid peptide; RGDS, arginine-glycine-aspartic acid-serine peptide; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

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renders platelets nonfunctional toward low-thrombin doses (0.05−0.2 unit/mL) and other agonists. A decrease in aggregation correlates with a lack of GPIIb-IIIa activation (Sakon et al., 1993). But the molecular mechanism(s) by which these enzymes regulate the responsive state of platelets is (are) not known. Their activities are required for ligand-induced phosphatidylinositol (PI) hydrolysis and calcium fluxes (Lerea, 1992; Murata et al., 1993; Koike et al., 1994), and are involved in the resensitization of the receptor (Brass, 1992). It is therefore not surprising that secretion and aggregation responses are inhibited by PP1/PP2A antagonists. Interestingly, the effects of calyculin A and okadaic acid on the first wave of signaling events induced by thrombin, e.g., PI responses and Ca^{2+} fluxes, can be overcome by the addition of higher thrombin doses (Lerea, 1992). The purpose of the present study was to determine whether events that normally follow aggregation are sensitive to these phosphatase inhibitors. The results demonstrate that inhibition of PP1/PP2A blocks aggregation-induced rearrangement of the cytoskeleton.

MATERIALS AND METHODS

Materials. Highly purified human thrombin (2694 units/mL) was a gift from Dr. Walter Kisiel, University of New Mexico. Prostaglandin E1 (PGE1), apyrases, leupeptin, aprotinin, phenylmethanesulfonyl fluoride, Triton X-100, arginine-glycine-aspartic acid (RGD) and arginine-glycine-aspartic acid-serine (RGDS) peptides, sodium vanadate, and anti-vinculin monoclonal antibody were from Sigma, and acrylamide, *N,N'*-methylenebis(acrylamide), and HRP-conjugated goat anti-mouse and rabbit antibodies were from Bio-Rad. Amersham supplied the enhanced chemiluminescence reagents, and New England Nuclear supplied the [^{32}P]-phosphoric acid (in water) and [γ-^{32}P]ATP (3000 Ci/mmol). Calyculin A and okadaic acid were purchased from LC Services, Woburn, MA. Polyclonal anti-pp60$^{c\text{-}src}$ and a monoclonal antibody against the p85 subunit of PI3-kinase were purchased from UBI, Lake Placid, NY. SEW-8 (a polyclonal antibody against GPIIb), a polyclonal antibody against GPIIIa, and SEW 16 (a polyclonal antibody against PECAM) were kindly provided by Dr. Peter J. Newman, Southeastern Blood Center of Wisconsin, Milwaukee, WI.

Platelet Preparation. Human blood was drawn into vacutainer collection tubes containing acid/citrate/dextrose, and platelet-rich plasma (PRP) was obtained following centrifugation at 130*g* for 20 min at 21 °C. Platelets were removed from the PRP by centrifugation procedures as previously described (Lerea, 1992). Washed platelets were suspended in a modified Tyrode's buffer containing 5 mM Hepes, pH 7.4, 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5.5 mM glucose (suspension buffer) at a concentration of 2×10^9/mL.

Platelet Aggregation. Aggregation measurements were conducted at 37 °C in siliconized cuvettes using a Chronolog lumiaggregometer. Platelet suspensions (6×10^8/mL in a final volume of 300 μL) were preincubated with buffer/vehicle (0.1% DMSO) or calyculin A (1−2 μM) prior to the addition of thrombin. For convenience, buffer/vehicle-treated platelets will be referred to as control platelets. Aggregation was allowed to proceed with constant stirring at 1000 rpm.

Isolation of Platelet Cytoskeleton. Aggregation reactions were quenched by the addition of 100 μL of ice-cold cytoskeletal lysis buffer containing 4% Triton X-100, 80 mM Tris-HCl, pH 7.5, 560 mM NaCl, 20 mM EDTA, 1.6 mM sodium vanadate, 40 μg/mL aprotinin, 4 mM phenylmethanesulfonyl fluoride, and 40 μg/mL leupeptin. The lysates were subjected to centrifugation at 14000*g* for 4 min at 4 °C, and the Triton-insoluble material (cytoskeleton) was washed in a 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.4 mM sodium vanadate. The pellets were either solubilized in SDS sample buffer or suspended in wash buffer for lipid and tyrosine kinase assays as previously described (Zhang et al., 1992; Lerea et al., 1989). The supernatants were further subjected to centrifugation at 100000*g* for either 2.5 h using a Ti 60 rotor or 14.5 h using a TLA 100.4 rotor in order to isolate membrane skeletons (Fox et al., 1993).

Western Analysis of Cytoskeletal Proteins. Cytoskeletal proteins were separated on 10% SDS−polyacrylamide gels followed by transfer onto nitrocellulose for 1 h at 100 V. For use with anti-src (1:600), anti-vinculin (1:4000), and anti-p85 (1:1000) antibodies, the nitrocellulose was blocked with TTBS (Tween-20-containing Tris-buffered saline) containing 5% nonfat dried milk. The membranes were subsequently incubated for 1−2 h with 5% milk containing the indicated antibody solutions. For use with anti-GPIIb (20 μg/mL), anti-GPIIIa (20 μg/mL), or anti-PECAM (20 μg/mL), antibodies, the nitrocellulose was blocked with TBS containing 3% milk plus 2% bovine serum albumin. These membranes were subsequently incubated for 1 h with 2% albumin containing antisera against GPIIb, GPIIIa, or PECAM. The membranes were then incubated with HRP-coupled secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG) and developed using the ECL detection kit.

Measurement of [^{32}P]Phosphoproteins. Platelets were suspended in modified Tyrode's buffer at a concentration of 7×10^8/mL and were incubated with [^{32}P]orthophosphate (0.1 mCi/mL) for 2 h at 37 °C. Platelets (300 μL) were treated with vehicle (0.1% DMSO) or calyculin A (2 μM) for 3 min. Reactions were quenched by addition of 100 μL of 4× cytoskeletal lysis buffer and cytoskeletal and membrane skeletal fractions isolated as described above. Proteins were separated by two-dimensional polyacrylamide gel electrophoresis as previously described with one exception, ampholines with pH ranges of 3−10, 5−7, and 6−8 (1:1:1) were used (Lerea, 1991). *In vitro* phosphorylation studies were conducted as previously described using membrane skeletal fractions instead of whole lysates (Lerea, 1992).

RESULTS AND DISCUSSION

Okadaic acid and calyculin A have been shown to render platelets nonfunctional in response to 0.05−0.2 unit/mL thrombin. So far, all the published data generated can be interpreted by invoking a phosphatase-sensitive step early in the cascade of events triggered by thrombin as well as other agonists. This interpretation is consistent with the observations that calyculin A, okadaic acid, and tautomycin inhibit PI metabolism and calcium fluxes induced by low thrombin doses. A decrease in the activation of GPIIb-IIIa has also been observed, which confirms that these early events are needed for fibrinogen binding. Treatment of platelets with 1−2 μM calyculin A inhibited the ability of thrombin (0.2 unit/mL) to induce aggregation (Figure 1). Calyculin A itself induces a shape change response, in

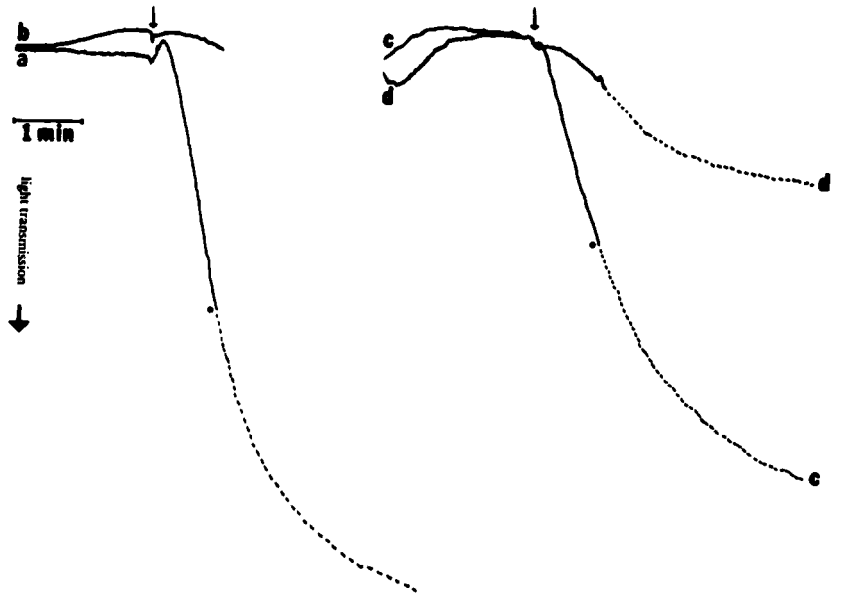

FIGURE 1: Aggregometer tracings of washed platelets preincubated with and without calyculin A. Washed platelets were added to a siliconized cuvettes and pretreated with either vehicle (curve a) or 1 μM calyculin A (curves b, c, d) for 2 min prior to stimulation with either 0.2 unit/mL (curves a and b) or 1 unit/mL (curves c and d). In addition, platelets suspensions used to generate curve d were pretreated with RGD (4 mM) in addition to calyculin A. With respect to curve d, identical results were obtained using RGDS (200 μM) (not shown). The extent of aggregation obtained by 1 min is indicated by the asterisks.

agreement with Yano et al. (1994), who employed electron microscopy to show calyculin-induced changes in platelet morphology. The ability of calyculin A or okadaic acid to block aggregation is dependent on the dose of the inhibitors (Karaki et al., 1989) and of thrombin (Figure 1). In the present study, 1−2 μM calyculin A inhibited greater than 95% of the aggregation induced by thrombin concentrations up to 0.2 unit/mL (n = 16). This inhibition was overcome by the addition of 1 unit/mL thrombin (curve c). This observed aggregatory response was 60−80% of the maximum seen with control platelets treated with the lower amount of thrombin (n = 14). Similar observations were found using 5 μM okadaic acid (not shown, n = 10). Similar observations were also demonstrated using platelets isolated by gel filtration procedures (not shown). Aggregation in the presence of 1−2 μM calyculin A was sensitive to RGD peptide (curve d) and 200 μM RGDS peptide (not shown), suggesting that it was mediated through GPIIb-IIIa and not through GP1b. This is reasonable since Ca^{2+} fluxes and protein kinase C activity [modulators of GPIIb-IIIa activation (Shattil et al., 1992)] increase in calyculin-treated platelets following the addition of high thrombin doses (Lerea, 1992). Moreover, ristocetin-induced aggregation is not blocked by 2 μM calyculin A (not shown).

In platelets, aggregation initiates signaling events and a reorganization of the platelet cytoskeleton. The redistribution of molecules to the Triton X-100-insoluble cytoskeletal fraction was monitored by SDS−PAGE followed by either Coomassie blue staining (Figure 2A) or Western (Figure 2B) analysis. For all samples, the extent of platelet aggregation was measured directly using a lumiaggregometer. For studies illustrated in Figure 2, aggregation reactions proceeded for 1 min (see Figure 1, asterisks) and were abruptly stopped directly in the cuvette by addition of Triton-lysis buffer. Coomassie blue profiles of proteins associated with the Triton-insoluble cytoskeletal fraction isolated from resting (Figure 2A, lane 1) and activated platelets (Figure 2A, lane 2) were significantly different: In particular, the cytoskeletons isolated from activated platelets contained proteins that migrated to the positions of actin-binding protein, talin, vinculin (which is weakly detected by Coomassie blue staining), and α-actinin. Pretreatment of platelets with 1−2 μM calyculin A blocked the ability of 0.2 unit/mL thrombin to induce such changes (lane 3, n = 4). Similar results were obtained using 5 μM okadaic acid (not shown). At these concentrations, both phosphatase inhibitors eliminated aggregation induced by low thrombin doses (Figure 1, curve b, and data not shown). Thus, the effect of these agents on the redistribution of vinculin, talin, and α-actinin is consistent with previous observations that the association of these molecules with the cytoskeletal fraction is proportional to the aggregatory response (Kouns et al., 1991; Fox et al., 1993). It is feasible that these observed affects are linked to the inhibitory effect of calyculin A and okadaic acid on PI metabolism and the subsequent activation of protein kinase C, a modulator of GPIIb-IIIa activation (Shattil et al., 1992).

The inhibitory effects of okadaic acid and calyculin A on PI metabolism, Ca^{2+} fluxes, and dense granule secretion (Lerea, 1992) and on aggregation (this study, Figure 1) are overcome by addition of high-thrombin doses. In contrast, actin-binding protein, talin, vinculin, and α-actinin were not detected when the cytoskeletal fractions of these aggregated platelets were analyzed by Coomassie blue staining (Figure 2A, lane 4). These findings suggests that aggregation-induced redistribution of proteins to the cytoskeleton is blocked by protein phosphatase inhibitors. This was confirmed by Western analysis (Figure 2B). In these studies, the presence of GPIIb, vinculin, PI3-kinase, and $pp60^{c\text{-}src}$ was measured in cytoskeletal fractions isolated from resting platelets (lanes 1), 0.2 units/mL thrombin-activated platelets (lanes 2), or calyculin A-treated platelets incubated with either 0.2 unit/mL (lanes 3) or 1 unit/mL (lanes 4) thrombin (n = 6−8): The extent of aggregation observed for each of these samples is shown in Figure 1. In agreement with previous studies (Zhang et al., 1992; Fox et al., 1993), each of these molecules associated with the cytoskeleton following

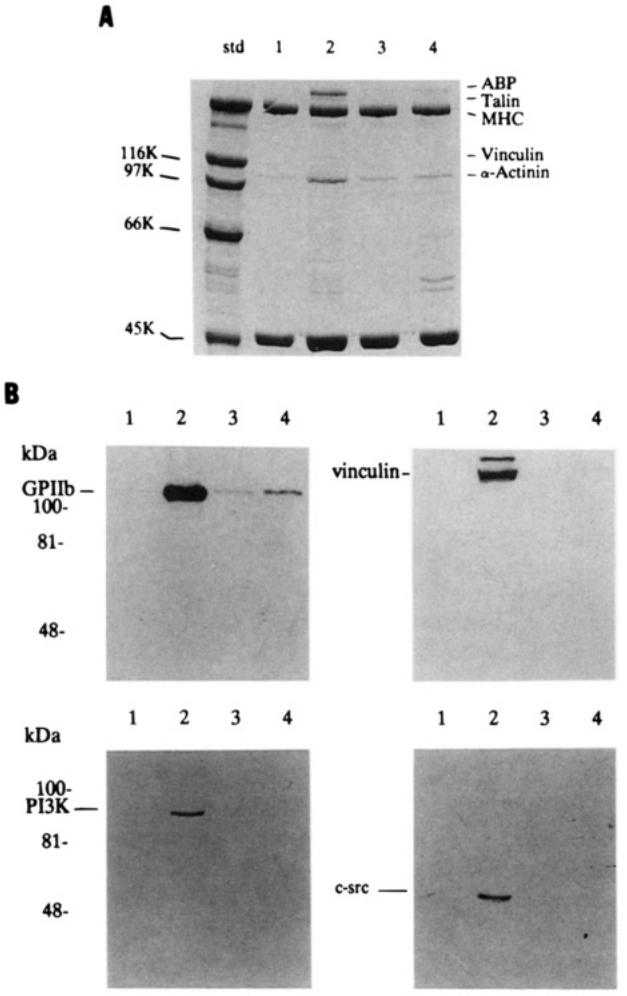

FIGURE 2: Analysis of proteins associated with platelet cytoskeleton. Panel A: Platelets were pretreated with vehicle (lanes 1 and 2) or with calyculin A (lanes 3 and 4) for 2 min prior to treatment with buffer (lane 1) or 0.2 unit/mL (lanes 2 and 3) or 1 unit/mL (lane 4) thrombin. The 14000*g* cytoskeletal fractions were solubilized with SDS sample buffer and electrophoresed using 10% SDS−polyacrylamide gel electrophoresis. Proteins were detected by Coomassie blue staining. The lane labeled std shows the position of Bio-Rad's high molecular weight standards. Panel B: Platelets were pretreated with vehicle (lanes 1 and 2) or with calyculin A (lanes 3 and 4) for 2 min prior to treatment with 0.2 unit/mL (lanes 2 and 3) or 1 unit/mL (lane 4) thrombin. The 14000*g* cytoskeletal fractions were prepared and proteins separated by SDS gel electrophoresis. The association of GPIIb, vinculin, the 85 kDa subunit of PI3-kinase, and pp60$^{c\text{-}src}$ was detected by Western analysis.

aggregation of control platelets (lanes 2). Also in agreement with previous reports, in the absence of aggregation, the association of these molecules with the cytoskeleton was not found (lanes 3). Consistent with the results shown in Figure 2A, these molecules were barely detected associated with the cytoskeleton in calyculin A-treated platelets treated with 1 unit/mL thrombin (lanes 4), although substantial aggregation occurred. Moreover, association of these proteins with the cytoskeletal fraction did not increase if aggregation was allowed to proceed to maximal levels (not shown). Similar results were observed when the amounts of GPIIIa and PECAM were measured by Western analysis (not shown, $n = 3$). The inability to detect aggregation-induced interactions between the cytoskeleton and GPIIb-IIIa, vinculin, PI3-kinase, and the pp60$^{c\text{-}src}$ does not reflect the instability of the cytoskeletal complexes (Bertagnolli & Beckerle, 1993); identical results were obtained when 5 mM EDTA/5 mM EGTA was added to aggregated platelet suspensions prior to addition of the Triton-lysis buffer (not shown, $n = 2$).

In agreement with the above observations, PI3-kinase activity was detected only in cytoskeletal fractions isolated from thrombin-activated control platelets (not shown, $n = 2$). Protein tyrosine kinase activity in each of the cytoskeletal fractions was also measured using RR-src as substrate. When kinase activity was measured in the cytoskeleton from resting platelets, only 0.4 pmol of phosphate was incorporated in 10 min. Following activation, this value increased to 25 pmol. The addition of 1 unit/mL thrombin to calyculin A-treated platelets did increase the amount of cytoskeletal-associated tyrosine kinase activity (to a value of 8.5 pmol incorporated/10 min) over that found associated with the cytoskeletons of resting platelets; presumably, the increase in activity reflects the presence of other cytoskeletal kinases, e.g., pp72^{syk} and pp62$^{c\text{-}yes}$; however, this needs to be verified.

The results of these experiments indicate that calyculin A inhibits aggregation-induced cytoskeletal rearrangements. The aggregatory response of calyculin A-treated platelets could reach as high as 80% of that seen with control platelets, but essentially a complete inhibition of aggregation-induced rearrangement of the cytoskeleton was observed. The mechanism by which phosphatase inhibitors affect platelet cytoskeleton is unclear. Recently, it has been suggested that actin-dependent associations are needed for aggregation-induced signaling. Pretreating platelets with cytochalasin D does not significantly alter the aggregation response toward thrombin, but it does block aggregation-induced actin polymerization and subsequent signaling (Huang et al., 1993; Lipfert et al., 1992). These authors propose a model in which the assembly of the platelet actin cytoskeleton is required for aggregation-induced signaling. Interestingly, in the present study, we routinely observed that the cytoskeletal aggregates that formed from aggregated-control platelets were larger than those which formed from aggregated calyculin A-treated platelets. Together, these results suggest that calyculin A may directly affect cytoskeletal reorganization.

The underlying mechanism(s) by which calyculin A or okadaic acid regulates cytoskeletal association of structural and signaling molecules is (are) not understood. Presumably, calyculin A-dependent phosphorylations are responsible for the effects. Essentially, nothing is known about the identity or location of proteins that become phosphorylated in calyculin A-treated platelets. Candidates for such substrates include talin (Murata et al., 1995) and an uncharacterized 50 kDa protein (p50). The addition of phosphatase inhibitors to platelets causes an increase in phosphorylation of p50, a protein that migrates in a two-dimensional gel system as a series of phosphorylated spots at approximately pH 6.2−6.7 and 50K molecular weight (Figure 3, panels A and B; Lerea, 1991). Using procedures to isolate various subfractions of the platelet cytoskeleton, this 50 kDa phosphoprotein was routinely recovered in the detergent-soluble fraction following the 14000*g* centrifugation spin, and was not associated with the low-speed cytoskeletal fraction (Figure 3, panel C). Interestingly, this p50 is localized to the membrane skeleton (Figure 3D, $n = 4$), which sediments at 100000*g* (Fox et al., 1993). This places the phosphorylated form of the 50 kDa protein in a position to regulate association of proteins to cytoskeletal structures. Two-

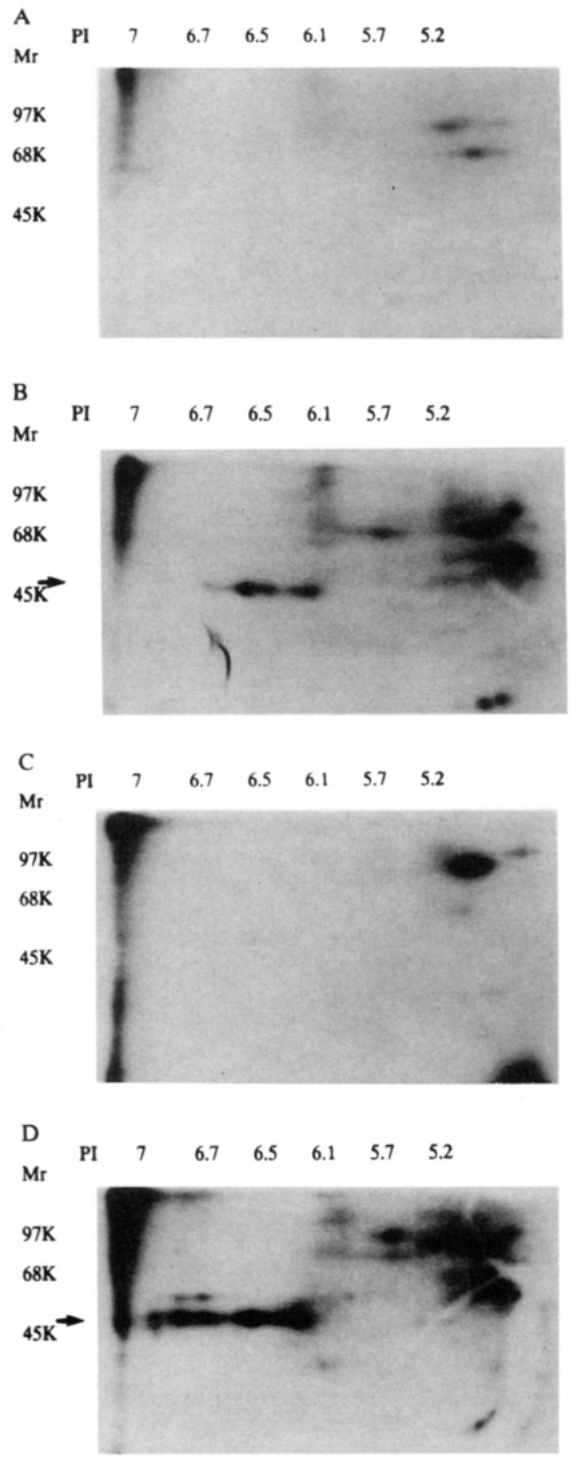

FIGURE 3: Subcellular localization of ^{32}P-labeled p50. Platelets were labeled with [^{32}P]orthophosphate as described under Materials and Methods. Platelets were treated for 2 min with either buffer (panel A) or 2 μM calyculin A (panels B−D). Panels A and B: Reactions were stopped by the addition of IEF gel sample buffer. Panels C and D: Reactions were quenched by the addition of Triton X-100 lysis buffer, and cytoskeletal (panel C) and membrane skeletal (panel D) structures were isolated and suspended in IEF gel sample buffer. Phosphoproteins were separated by two-dimensional gel electrophoresis and detected by autoradiography using Kodak X-AR film.

dimensional gel electrophoresis of p50 enriched in the membrane skeleton revealed a pattern of spots similar to that observed of p50 from calyculin A-treated platelets (panels B and D). However, additional spots of 50 kDa with greater basic isoelectric points were visualized in the membrane

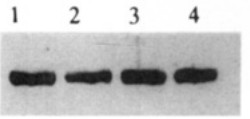

FIGURE 4: Association of GPIIb membrane skeletons isolated from buffer- and calyculin A-treated platelets. In cuvettes, platelets were pretreated with vehicle (lanes 1 and 2) or calyculin A (lanes 3 and 4) prior to addition of buffer (lane 1), 0.2 unit/mL thrombin (lanes 2 and 3), or 1 unit/mL (lane 4) thrombin. Membrane skeletons were isolated and proteins separated by SDS−polyacrylamide gel electrophoresis. Western analysis was conducted using anti-GPIIb antiserum.

skeleton. Previously, tryptic phosphopeptide map analyses indicate that the multiple phosphorylated spots shown in panel B represent multiple phosphorylation of one protein (Lerea, 1992). Thus, it can be postulated that the additional heterogeneity of spots observed in the membrane skeleton most likely reflects less phosphorylated forms of a common protein. It is not known whether the increased heterogeneity becomes evident because an enriched subcellular fraction was used or because dephosphorylation was occuring during preparation of the membrane skeleton.

The membrane skeletal-associated 50 kDa protein was phosphorylated only in platelets that were treated with calyculin A (not shown). The phosphatase inhibitor itself does not cause the disassembly of the membrane skeleton. The amount of GPIIb (Figure 4, $n = 2$) and vinculin (not shown) associated with the membrane skeleton was similar in resting platelets (Figure 4, lane 1) and calyculin A-treated platelets (Figure 4, lanes 3 and 4). A slight decrease was observed only in the membrane skeletal fraction isolated from thrombin-treated platelets (Figure 4, lane 2), most likely reflecting a translocation to the cytoskeletal fraction.

The above data support the notion that the phosphorylated form of p50 is part of the membrane skeleton. To determine whether the dephosphorylated form resides in this cellular compartment, phosphorylation assays were conducted using a cell-free system as previously described (Lerea, 1992). In these studies, membrane skeletons were isolated from resting platelets and tested for calyculin A-induced phosphorylation of p50. The addition of calyculin A resulted in the increase in its phosphorylation state (Figure 5, $n = 3$). The data strongly argue in favor of p50 being associated with this subcellular fraction. It is also noteworthy that the protein kinase and phosphatase activities modulating the phosphorylation state of p50 also reside in this fraction. In this respect, approximately 20% of the phosphorylase phosphatase activity was found to be associated with subcellular structures in platelets (Lerea, 1991).

The identify of p50 is still not known. Interestingly, it shares several features that are intrinsic to the 50 kDa platelet substrate (VASP) of the cAMP- and cGMP-dependent protein kinases (Horstrup et al., 1994): (i) Both molecules have molecular weights of 50K in the phosphorylated state; (ii) the extent of phosphorylation of both proteins correlates with inhibition of platelet activation; (iii) phosphorylation of VASP correlates with inhibition of GPIIb-IIIa activation (Horstrup et al., 1994), whereas p50 phosphorylation correlates with inhibition of aggregation-induced signaling; and (iv) both proteins are associated with cytoskeletal structures [this study and Reinhard et al. (1992)]. Taken together, these observations raise the intriguing possibility that p50 and VASP are similar proteins. The observation that these two

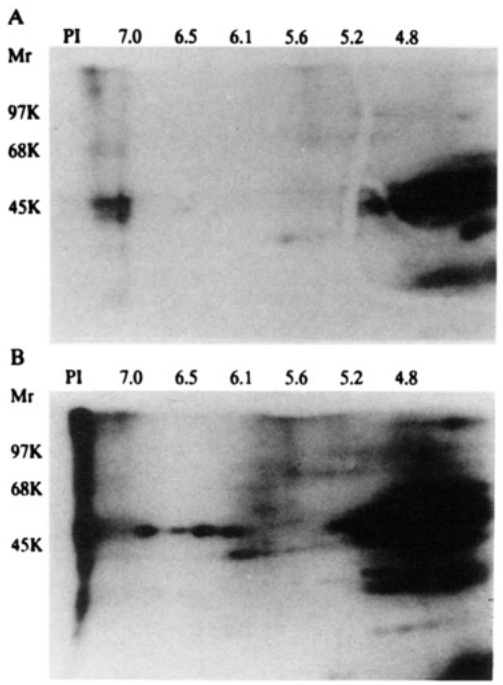

FIGURE 5: *In vitro* phosphorylation of membrane skeletal proteins. Membrane skeletons were isolated from resting platelets and incubated in the presence of 50 μM [γ-^{32}P]ATP and buffer (panel A) or 2 μM calyculin A (panel B). Reactions were quenched by the addition of IEF gel buffer, and the proteins were separated by two-dimensional PAGE.

proteins do not always comigrate on two-dimensional gels (Lerea, 1991) may reflect differences in the degree or site-(s) of phosphorylation following treatment with okadaic acid/calyculin A and cAMP elevating agents. It is significant in this regard that the calyculin A-induced phosphorylation of p50 is not mediated by cyclic nucleotide-dependent protein kinases (Lerea, 1992).

In summary, delineating the sequence of events where protein phosphatases exert an affect in platelets has been difficult since high levels of agonists appear to bypass phosphatase-sensitive sites, at least with respect to secretory and aggregatory responses. The present findings indicate that protein phosphatases are crucial for aggregation-induced signaling events in platelets. Inhibitors of types 1 and 2A enzymes dissociate cytoskeletal rearrangement from an aggregatory response. Previously, a close relationship has been shown to exist between the effect of calyculin A and the phosphorylation of a 50 kDa protein (Lerea, 1992). The importance of this phosphorylation event with respect to inhibition of cytoskeletal rearrangements is now emphasized by finding p50 associated with the membrane skeletal compartment.

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