

Reciprocal Transmembranous Receptor–Cytoskeleton Interactions in Concanavalin A-activated Platelets

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ABSTRACT Concanavalin A (Con A) has been used to activate platelets, inducing a specific interaction between the glycoprotein IIb-IIIa complex and the cytoskeleton of the activated platelet. In agreement with this, we have shown that Con A activates human platelets, initiating phosphorylation, secretion, and cytoskeletal formation. Con A and cytochalasin B were used to demonstrate a reciprocal interaction of the glycoprotein complex with the platelet cytoskeleton. Additionally, we have shown that a similar reciprocity is provided by the multivalent fibrin–fibrinogen platelet interaction found in the thrombin-induced clot. Con A differs from other activators in precipitating an apparent cytoskeletal core despite a complete inhibition of platelet activation by prostaglandin E₁. We suggest, from this result, that Con A may be cross-linking a membrane-associated cytoskeletal complex present in the unactivated platelet.

Concanavalin A (Con A),¹ A multivalent lectin, has been used to demonstrate transmembranous receptor–cytoskeleton interactions in fibroblasts (1), lymphocytes (2), and adenocarcinoma cell lines (3). In addition, Con A binds to platelet surface glycoproteins, especially each component of the glycoprotein IIb-IIIa complex (4). The tetrameric Con A molecule, as well as other multivalent lectins, also activates platelets by an as yet unknown mechanism (5). It has been proposed that Con A activation leads to cytosolic calcium elevation with subsequent protein phosphorylation and cytoskeletal core formation in the same manner as thrombin or arachidonate (6). Painter et al. (7) have reported that Con A induces a specific transmembranous interaction between the glycoprotein IIb-IIIa receptor complex and the cytoskeleton of the activated platelet. This interaction is more extensive than that seen with typical platelet activators in that 85–90% of the surface-labeled glycoprotein IIb-IIIa complex is retained in the Con A-induced cytoskeletal core, whereas thrombin-aggregated platelets retain only ~26% of this complex (8).

The terms contractile gel and pseudopodal cytoskeletons have been ultrastructurally defined as the dense microfilament network responsible for platelet secretory granule centralization and the parallel microfilament bundles observed in plate-

let pseudopodia (9). These cytoskeletal structures resist non-ionic detergent extraction and can be collected as cytoskeletal cores which, remarkably, retain the ultrastructural form observed in the intact platelet (8).

Our previous studies have shown that the contractile gel and pseudopodia can be separately assembled with unique cytoskeletal core components (9). In the presence of cytochalasin B (CB), the activated platelets form a contractile gel and secrete granules normally but have limited pseudopodal development, as observed by transmission electron microscopy. Although CB did not affect the extent of actin polymerization or actin-binding protein phosphorylation, we observe an almost complete loss of actin-binding protein and about half of the actin from the detergent extract. There was also a comparable loss of alpha-actinin and tropomyosin, which were not quantitated in this study. Under these conditions, the cytoskeletal core consisted of actin–tropomyosin, alpha-actinin, and an unreduced quantity of myosin.

Our conclusion from these studies was that CB was preventing the formation of long actin filaments which, when cross-linked by actin-binding protein, made up the pseudopodal cytoskeleton. The question of whether or not actin-binding protein was interacting with the nonsedimenting filamentous actin was not resolved in this study.

More recently, we have shown that the glycoprotein IIb-IIIa complex, which forms the fibrinogen binding site ex-

¹ *Abbreviations used in this paper:* Con A, concanavalin A; CB, cytochalasin B; PGE₁, prostaglandin E₁.

pressed upon platelet activation, is specifically linked to the CB-sensitive pseudopodal cytoskeleton (10). This conclusion is also based on the observation that retention of the glycoprotein IIb-IIIa complex in the detergent-insoluble core is not observed when platelets are activated in the presence of CB. Again, we attribute this to an inhibition by CB of long actin filament formation, since fibrinogen binding and platelet aggregation dependent on the glycoprotein IIb-IIIa complex were not blocked by CB. This left open the possibility that linkages between glycoprotein IIb-IIIa complexes and the nonsedimenting actin-binding protein-actin filaments still occurred but that such complexes were insufficiently cross-linked to precipitate after detergent extraction. Although the exact nature of this linkage and its importance to the expression of the fibrinogen binding site is unknown, we did determine in this study that receptor occupancy alone was insufficient to retain the complex in the isolated cytoskeletal core. Rather, a multivalent interaction with these surface receptors is required to stabilize their interaction with the cytoskeleton.

This entire cytoskeletal assembly process of actin polymerization and interaction with phosphorylated myosin and actin-binding protein can be completely reversed by agents that elevate platelet cytosolic cyclic AMP levels (11, 12). This oppositional control is most likely due to the lowering of cytosolic calcium levels elevated during activation (13). Moreover, these studies showed that the cytoskeletal assembly-disassembly cycles correlated completely with the reversal of platelet shape change, aggregation, and expression of the fibrinogen binding site.

In this study, we have used Con A and CB to demonstrate a reciprocal interaction of the glycoprotein IIb-IIIa complex with platelet pseudopodal elements. Moreover, using prostaglandin E₁ (PGE₁) and the reciprocal Con A-cytoskeleton interaction, we demonstrate a membrane-associated cytoskeletal complex that may be pre-organized in the unactivated platelet. Evidence is also presented that the physiological correlate to the multivalent binding of Con A may be the fibrin-fibrinogen-platelet interaction found in the forming thrombin-induced clot.

MATERIALS AND METHODS

Lyophilized human fibrinogen was purchased from Helena Laboratories, Beaumont, TX. [¹⁴C]5-Hydroxytryptamine (serotonin) and carrier-free Na¹²⁵I were obtained from Amersham, Radiochemical Center, Amersham, England. ³²P-Orthophosphate was obtained from New England Nuclear, Boston, MA. Sigma Chemical Co., St. Louis, MO, supplied the bovine serum albumin (BSA), Con A, Con A-Sepharose 4B-CL, Triton X-100, glucose oxidase, Sepharose 2B, PGE₁, and CB. Thrombin was a generous gift from Dr. Charles T. Esmon (Oklahoma Medical Research Foundation). Lyophilized lactoperoxidase was purchased from Calbiochem-Behring Corp., La Jolla, CA. Electrophoresis reagents and the high molecular weight standard protein mixture were purchased from Bio-Rad Laboratories, Richmond, CA.

Preparation of Gel-filtered Platelets: Blood, obtained by standard venipuncture techniques from normal drug-free human volunteers, was drawn into 0.1 vol of 100 mM citrate acid and 130 mM glucose (pH 6.5). Platelet-rich plasma was separated by centrifugation of the blood at 1,000 g for 3 min at room temperature, and platelets were pelleted by centrifugation as previously described (9). Platelets for surface iodination were resuspended in Tangen-HEPES (145 mM sodium chloride, 5 mM potassium chloride, 0.1 mM magnesium chloride, 0.05 mM calcium chloride, 5.5 mM glucose, and 15 mM HEPES, pH 7.4) with the addition of 1 μg/ml PGE₁. Platelet surface membrane proteins were radiolabeled with ¹²⁵I by lactoperoxidase-catalyzed iodination before gel filtration as previously described (10). Platelets for phosphorylation studies were resuspended in Tangen-HEPES buffer with 1 mg/ml BSA and allowed to incorporate ³²P-orthophosphate (1 mCi/ml) for 45 min at 37°C (9). Platelets for secretion studies were similarly resuspended and incubated with

0.05 μCi/ml [¹⁴C]serotonin added for the last 20 min. Residual plasma proteins and unincorporated label (¹²⁵I, ¹⁴C, or ³²P) were removed by gel filtration at 4°C on a Sepharose 2B column equilibrated and eluted with Tangen-HEPES-BSA (1 mg/ml). After gel filtration, the final platelet concentration was adjusted to 5 × 10⁸ platelets/ml and warmed to 37°C before experimentation. Serotonin secretion time courses were performed as previously described (9).

Preparation of Cytoskeletal Cores: Platelet aliquots were activated, with stirring at 37°C to give full aggregation, by Con A (100 μg/ml) or thrombin (1 U/ml) with and without pretreatment for 5–10 min with CB (5 μg/ml) or pretreatment with PGE₁ (1 μg/ml). In studies involving exogenous fibrinogen, human fibrinogen (purified and characterized as previously described [10]) in HEPES-buffered saline was added to a final concentration of 0.1 mg/ml before activation with thrombin. Cytoskeletal cores were extracted as described previously (9) and were pelleted from the extraction mixture by centrifugation at 10,000 g for 5 min at 0–4°C. Samples containing exogenous fibrinogen were subjected to two additional detergent washes before denaturation.

DNase I-Actin Inhibition Assays: Total actin content of whole platelet lysates and cytoskeletal cores was determined after depolymerization in guanidine buffer as previously described (9).

SDS Polyacrylamide Gel Electrophoresis: Cytoskeletal core pellets from 0.5 ml of platelets (5 × 10⁸/ml) were denatured with 0.5 ml of one-fold-concentrated denaturing solution consisting of 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 10% glycerol, 3 mM EDTA, 12 mM EGTA, 0.01% bromophenol blue, and 50 mM Tris-HCl (pH 6.8). A 0.4-ml aliquot of the supernatant samples was treated with 0.1 ml of a five-fold-concentrated denaturing buffer. Total platelet protein was prepared by mixing a 0.2-ml aliquot of the platelet suspension with 0.1 ml of the three-fold-concentrated denaturing solution. All samples were incubated at 100°C for 3 min and electrophoresed on 6–17.5% polyacrylamide gradient SDS slab gels prepared as described by Studier (14). Staining and autoradiography were performed as previously described (9, 10). A Bio-Rad high molecular weight standard (Bio-Rad Laboratories) consisting of myosin, beta-galactosidase, phosphorylase A, BSA, and ovalbumin was included on each gel. Laser densitometric scanning was done with an LKB 2202 ultrascan densitometer with an LKB 2220 recording integrator (LKB Instruments, Inc., Gaithersburg, MD).

Preparation of Samples for Electron Microscopy: Platelets were activated with Con A (100 μg/ml) with and without pretreatment with CB (5 μg/ml) and with and without pre-exposure to PGE₁ (1 μg/ml). Samples were stirred constantly in a Payton aggregometer (Payton Scientific Inc., Buffalo, NY) maintained at 37°C. Aliquots were removed at zero time, at 5 min, and at 10 min. The samples were fixed for 10–15 min at 37°C with an equal volume of 0.1% glutaraldehyde in White's saline (15). Samples were further fixed overnight at room temperature in 3% glutaraldehyde in White's saline, then for 90 min at 4°C in 1% osmium tetroxide in 15 mg/ml potassium ferricyanide. After fixation, the samples were washed twice with distilled water, then left overnight at 4°C in 3% uranyl acetate before dehydration through a graded series of ethanols and embedment in Spurr resin. After sectioning, samples were further stained using 0.2% lead citrate, then examined and photographed using a Philips 400 EM transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) at 80 kV.

RESULTS

Platelet Aggregation, Secretion, and Cytoskeletal Formation

Con A induces the formation of a complete cytoskeleton (Fig. 1, lane 3), similar to the thrombin-induced cytoskeleton (lane 2). The cytoskeletal core content of filamentous actin is slightly reduced for activation with Con A with respect to thrombin as assessed by DNase I inhibition assay (Table I). At 1 U/ml thrombin, maximal aggregation occurs between 2 and 3 min, while activation by Con A follows a slower time course with maximal aggregation between 7 and 8 min (Fig. 2). The secretion of serotonin is also delayed with a maximum of 40% secretion at 5 min with Con A in contrast to maximal secretion with thrombin which occurs between 30 and 60 s followed by a 10–20% re-uptake (Fig. 3).

When the cytosolic levels of cyclic AMP are elevated by pretreatment with PGE₁ or dibutyryl cyclic AMP sufficient to

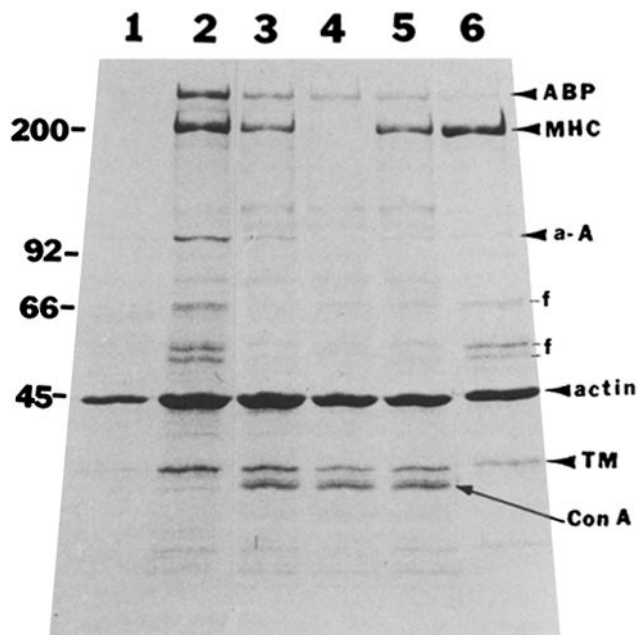


FIGURE 1 Polyacrylamide gradient-SDS gels of concanavalin A-activated platelets. Aliquots of ^{125}I -surface labeled-platelets were activated and cytoskeletal cores prepared as described in Materials and Methods. Equivalent aliquots of cytoskeletal core were subjected to electrophoresis on 6–17.5% polyacrylamide gradient-SDS slab gels, and the gels were stained with Coomassie Blue. The cytoskeletal cores are from: (1) control, unactivated platelets; (2) platelets activated with thrombin (1 U/ml); (3) platelets activated with Con A (100 $\mu\text{g}/\text{ml}$); (4) platelets pretreated with PGE_1 (1 $\mu\text{g}/\text{ml}$), then activated with Con A; (5) platelets pretreated with CB (5 $\mu\text{g}/\text{ml}$), then activated with Con A; (6) platelets pretreated with CB (5 $\mu\text{g}/\text{ml}$), then activated with thrombin (1 U/ml). The cytoskeletal components indicated are: actin-binding protein (ABP), myosin heavy chain (MHC), alpha-actinin (a-A), actin, and tropomyosin (TM). Also indicated are the fibrin (f) and concanavalin A (Con A) subunits.

TABLE I. Cytoskeletal Core Composition

Treatment	Actin**	ABP*	Myosin*	GPIIIa [§]
Controls	<10%	<5%	<5%	<3%
Thrombin	60–85	60–80	60–80	25 \pm 1
CB + thrombin	40–50	6–14	62–70	<3%
Con A	60–80	60–80	60–80	79 \pm 4
CB + Con A	42–45	27–37	29–35	84 \pm 5
Con A + PGE_1	40–45	28–42	3–9	84 \pm 5

* Determined by laser densitometry of Coomassie Blue-stained bands (ABP, 260-kD actin-binding protein; myosin 200-kD heavy chain) resolved on SDS slab gels ($n = 3$).

** Determined by DNase I inhibition assay ($n = 3$).

§ Determined by laser densitometry of ^{125}I -labeled autoradiograms (\pm SEM, $n = 5$).

† Controls include unactivated platelets with and without pretreatment with CB or PGE_1 , platelet lysates to which Con A or fibrinogen have been added before pelleting, and platelets pretreated with PGE_1 before addition of fibrinogen and thrombin.

block cytoskeletal formation by thrombin, the pseudopodal cytoskeletal elements (actin cross-linked by actin-binding protein and alpha-actinin) still form a precipitable matrix in the presence of Con A (Fig. 1, lane 4) with only slight reduction in the core content of actin. This actin in the core is most likely that filamentous component present in the resting platelet (typically in the 30–40% range) since no more than a 5–10% increase in filamentous actin occurs, as measured by

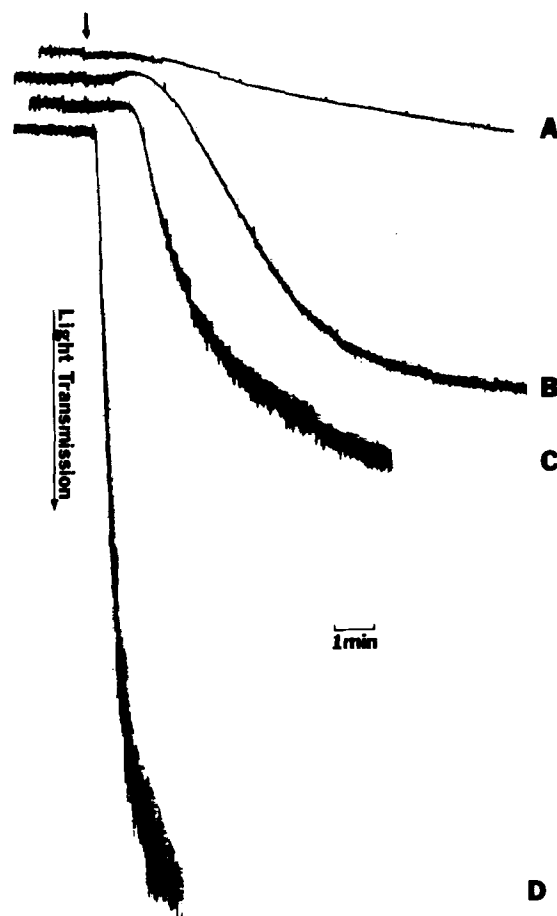


FIGURE 2 Aggregometer tracing of platelet aliquots. Tracing of aliquots of platelets (5×10^6 platelets/ml) were obtained as described in Materials and Methods. Platelets were activated with 100 $\mu\text{g}/\text{ml}$ Con A after pretreatment with: (A) 1 $\mu\text{g}/\text{ml}$ PGE_1 ; (B) no pretreatment; (C) 5 $\mu\text{g}/\text{ml}$ CB; or (D) 1 U/ml thrombin. Arrow indicates addition of activator.

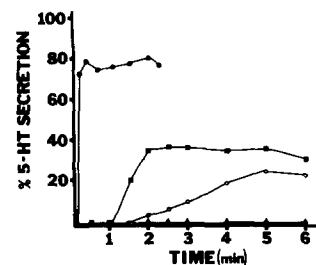


FIGURE 3 Secretion time course. Platelets were labeled with [^{14}C] 5-hydroxytryptamine as described in Materials and Methods. Samples were activated at timer zero with 1 U/ml thrombin (●); 100 $\mu\text{g}/\text{ml}$ Con A (○); pretreated with 5 $\mu\text{g}/\text{ml}$ CB for 15 min at 37°C, then activated at time zero with Con A (■); pretreated with 1 $\mu\text{g}/\text{ml}$ PGE_1 , for 5 min at 37°C before activation with Con A (▲).

DNase inhibition assays of whole platelet lysates. This precipitation of an apparent pseudopodal cytoskeleton occurs even though platelet activation is also largely suppressed in terms of aggregation (Fig. 2), secretion (Fig. 3), and actin-myosin interaction in the contractile gel (Fig. 1, lane 4).

Pretreatment with CB enhances the Con A-induced aggregation and secretory responses, as has been observed with other activators (9). CB pretreatment, which inhibits the formation of the long actin filaments necessary for pseudo-

podal formation, does not reduce the retention of pseudopodal elements (actin-binding protein and alpha-actinin) from the Con A-stimulated cytoskeletal core (Fig. 1, lane 5). This is in contrast to the thrombin-induced cytoskeleton in which pretreatment with CB completely releases the actin-binding protein and a significant quantity of the alpha-actinin from the cytoskeletal core (Fig. 1, lane 6). No additional precipitate, over control level, is observed (data not shown) if the Con A is added after lysis of the unactivated platelets. This is in agreement with the results of Painter et al. (7).

Electron Microscopy of Con A-activated Platelets

Electron micrographs of unactivated controls, with or without PGE₁ pretreatment, show discoidal platelets containing numerous randomly distributed granules (Fig. 4A). In agreement with the above results, at 5 min after stimulation with Con A, small aggregates of platelets exhibiting a loss of discoid shape, granule centralization, degranulation, and pseudopodal formation are evident (Fig. 4, B and C). As has been previously noted (5), the surface-connected canalicular system appears dilated and is more prominent than is typically observed with other activation conditions. Pretreatment with CB followed by Con A activation produces a similar picture of activated platelets but with somewhat fewer pseudopodal projections (not shown).

Platelets pretreated with PGE₁ and then exposed to Con A show some degree of cell-cell adherence by 10 min but do not show any significant loss of discoid shape, granule centralization, or degranulation (Fig. 4, D, E, and F).

Phosphorylation of Platelet Proteins

Activation of platelets by thrombin has been shown to induced rapid two- to threefold increases in phosphorylation of actin-binding protein (16) and myosin light chain (17), as well as of a cytosolic 47,000-D protein (18) designated 40P (5–10-fold) with peak ³²P-labeling between 30 and 60 s. Activation with Con A produces similar increases in phosphorylation of myosin light chain and 40P (Fig. 5A) (however, the phosphorylation of actin-binding protein is less than two-fold). The time course, like the aggregation and secretion response, is slower with peak ³²P-labeling occurring between 5 and 6 min. Pretreatment with CB followed by Con A activation does not alter the ³²P-labeling pattern (data not shown). Pretreatment with PGE₁ completely blocks the ³²P-labeling of the myosin light chain (Fig. 5B). The actin-binding protein, along with 24- and 22-kD peptides (19), is phosphorylated by PGE₁ independent of activation as previously reported for PGI₂ or dibutyl cyclic AMP (11).

Cytoskeletal Core Content and Retention of Glycoprotein IIb-IIIa

Quantitation of the cytoskeletal cores of the activated platelets by DNase I inhibition assay for actin content and laser densitometry of the Coomassie Blue-stained gels for myosin and actin-binding protein content is summarized in Table I. The effect of pretreatment with PGE₁ on Con A activation is a slight reduction in cytoskeletal core actin content with a significant decrease in myosin. Pretreatment with CB produces the same slight decrease in actin content with either Con A or thrombin activation. The release of actin-binding protein from the core, under the influence of CB, is far greater

with thrombin activation than with Con A.

Retention of the ¹²⁵I-labeled glycoprotein IIb-IIIa complex in the cytoskeletal cores was assessed by autoradiography and laser densitometry. The retention, expressed as a percentage of the whole platelet, was calculated using the glycoprotein IIIa band as a marker for the complex, since it is more heavily labeled and easier to quantitate. In all platelet samples activated with Con A, irrespective of pretreatment, the retention was in excess of 80%. Thrombin-activated platelet cores contained 25% of the total glycoprotein IIb-IIIa complex, and pretreatment with CB reduced the core content to control levels. Similar data was previously reported for arachidonate-activated platelets, with or without pretreatment with CB (10).

Cytoskeletal Formation and Glycoprotein IIb-IIIa Retention in the Presence of Fibrinogen

To investigate whether the multivalent surface binding provided by Con A might be responsible for retention of the pseudopodal elements in the presence of CB, platelets were activated with thrombin in the presence of 0.1 mg/ml exogenous fibrinogen (0.5 kD for fibrinogen-receptor binding). In addition to activating the platelets, thrombin will convert the soluble fibrinogen into the insoluble fibrin network. The fibrin network can then provide multiple sites for glycoprotein IIb-IIIa interaction. The cytoskeletal composition of ¹²⁵I-surface-labeled platelets activated with and without pretreatment with CB and in the presence or absence of exogenous fibrinogen are shown in Fig. 6A. The autoradiogram of the retained surface-labeled protein is shown in Fig. 6B. The cytoskeletal core from platelets activated with Con A (100 µg/ml) is shown in lane 1. In addition to retaining 79 ± 4% of the glycoprotein IIb-IIIa complex, other glycoproteins, including one ~140,000 D (possibly glycoprotein Ib), can be detected in these cores. Thrombin activation in the absence (lane 2) and presence of exogenous fibrinogen (lane 3) yield the same core compositions. However, the retention of the glycoprotein IIb-IIIa complex in the core is enhanced by the presence of exogenous fibrinogen (Fig. 6B, lanes 2 and 3). Quantitation by laser densitometry indicated that without exogenous fibrinogen, 25 ± 1% of the surface-labeled glycoprotein IIa is retained in the cytoskeletal core, whereas in the presence of exogenous fibrinogen, 36 ± 3% of the glycoprotein IIIa is retained. CB-pretreated platelets activated without exogenous fibrinogen do not contain a significant proportion of actin-binding protein (lane 4), and the core content of glycoprotein IIIa is reduced to control levels (<3%). In the presence of fibrinogen, the cytoskeletal cores from CB-pretreated platelets activated by thrombin contain a significant portion of actin-binding protein (lane 5), and the retention of the glycoprotein IIIa in the core is increased 10-fold (31 ± 3%).

As with Con A, the addition of a similar amount of fibrinogen to the lysates of unactivated platelets does not increase the levels of any retained core component, including the glycoprotein IIb-IIIa complex (Table I). This control demonstrates the need for an intact membrane surface-cytoskeletal system for these interactions to take place. In addition, platelets, exposed to thrombin and fibrinogen after pretreatment with PGE₁ to block activation, do not show increased retention of cytoskeletal core components. This latter finding demonstrates the need for platelet activation expressing the fibrinogen binding site in order for the transmembrane cytoskeletal interaction with the fibrin network to occur.

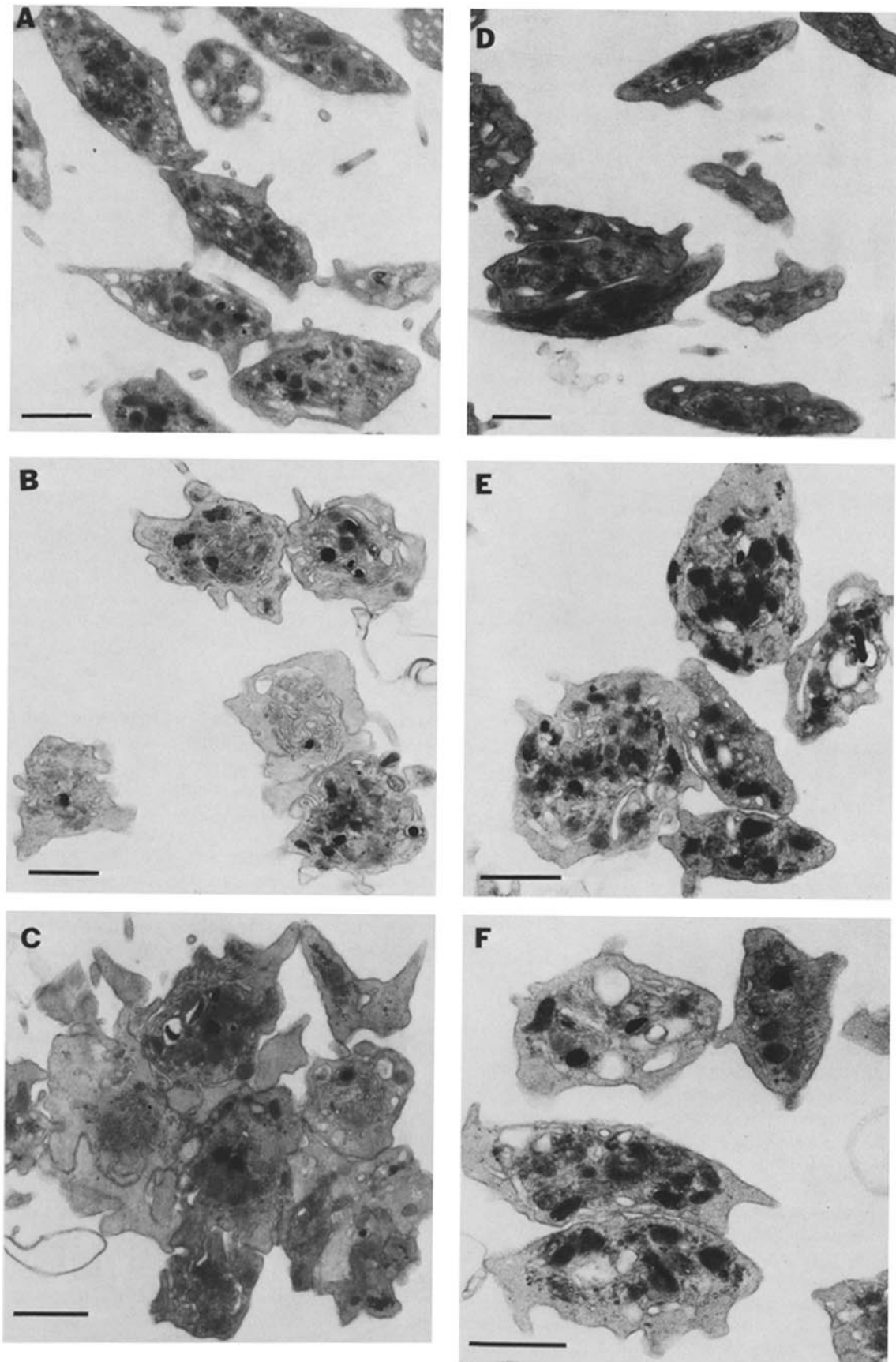


FIGURE 4 Electron micrographs of Con A activated with platelets. Platelets were either unactivated controls (A); activated with 100 $\mu\text{g}/\text{ml}$ Con A for 5 min (B); activated with 100 $\mu\text{g}/\text{ml}$ Con A for 10 min (C); or pretreated for 10 min with 1 $\mu\text{g}/\text{ml}$ PGE₁, then activated with Con A for 10 min (D, E, and F). Bar, 1 μm .

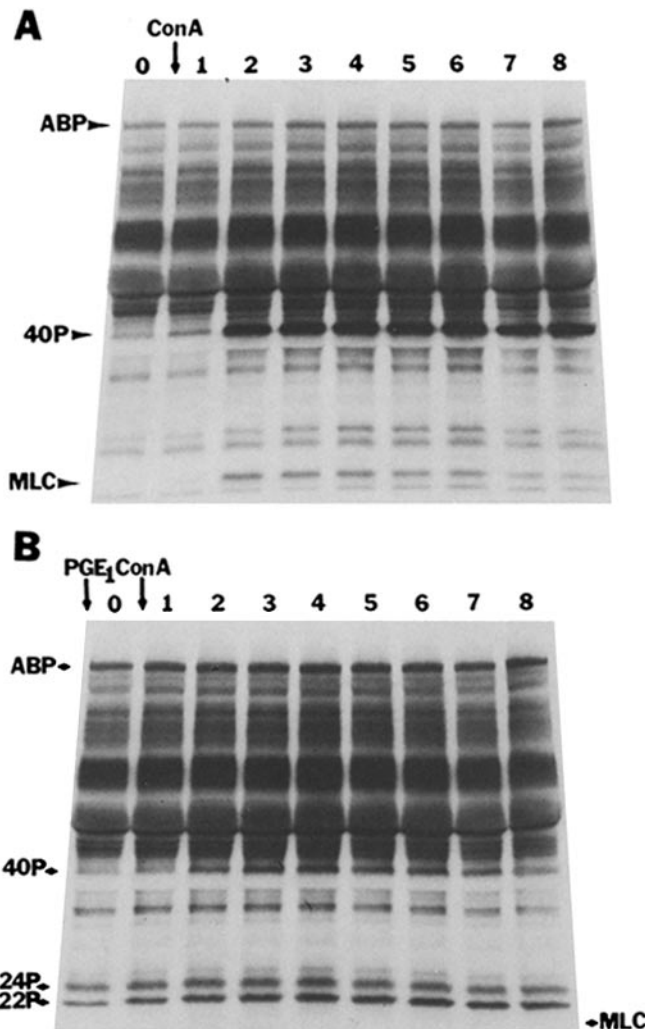


FIGURE 5 Autoradiograms of ^{32}P -labeled platelets. ^{32}P -labeled platelets were activated with 100 $\mu\text{g}/\text{ml}$ Con A (A) or pretreated with 1 $\mu\text{g}/\text{ml}$ PGE_1 , then activated with 100 $\mu\text{g}/\text{ml}$ Con A (B). Whole platelet aliquots were denatured at time (min) indicated. Samples were electrophoresed and autoradiographed as described in Materials in Methods. (ABP) Actin-binding protein; (40P) 47-kD protein; (MLC) myosin light chain; (22P) 22-kD phosphoprotein; (24P) 24-kD phosphoprotein.

DISCUSSION

The activation of platelets by Con A has been suggested to be an analogue of the normal receptor-mediated activation pathway (6). In agreement with this, we have shown that Con A activates human platelets, initiating phosphorylation, secretion, and the formation of a cytoskeletal structure that contains all the structural elements found in the thrombin or arachidonate-activated platelet.

Platelet stimulus-response coupling is most likely mediated by increased phospholipid metabolism (20). The subsequent phospholipase C-mediated formation of diacylglycerol leads to activation of protein kinase C (21, 22). Protein kinase C phosphorylation of actin-binding protein (23) may regulate its cross-linking of filamentous actin into a pseudopodal cytoskeleton (16, 24). At the same time, formation of thromboxane A_2 from the arachidonic acid metabolic pool, lysophosphatidic acid, and/or inositol 1, 4, 5-trisphosphate mobilizes calcium from the intracellular pool (25–28). Cytosolic

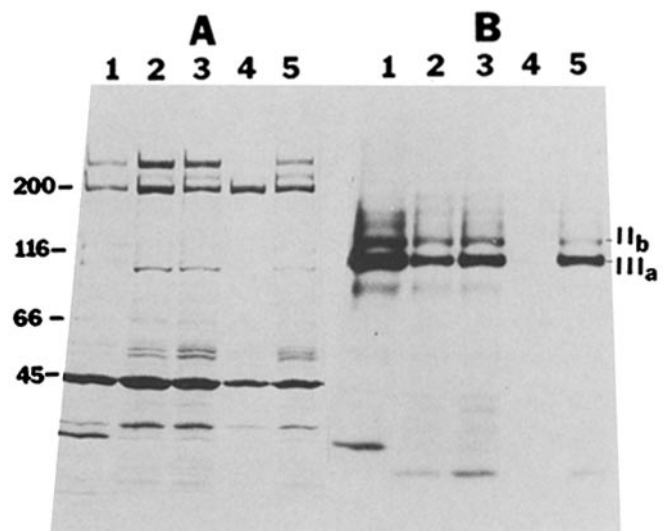


FIGURE 6 Polyacrylamide gradient-SDS gels of platelet cytoskeletons and retention of surface-labeled membrane proteins. Equivalent aliquots of platelets were subjected to electrophoresis on 6–17.5% polyacrylamide gradient-SDS slab gels, and the gels were stained for protein with Coomassie Blue (A). Cytoskeleton was from: (1) platelets activated with 100 $\mu\text{g}/\text{ml}$ Con A; (2) after activation with 1 U/ml thrombin; (3) activation with 1 U/ml thrombin in the presence of 0.1 mg/ml fibrinogen; (4) pretreated with CB, activated with 1 U/ml thrombin; (5) pretreated with CB, activated with 1 U/ml thrombin in the presence of 0.1 mg/ml fibrinogen. B is the autoradiogram of the protein-stained gel in A showing retention of the ^{125}I -surface labeled proteins. II_b, glycoprotein II_b; III_a, glycoprotein III_a.

calcium-mediated phosphorylation of the myosin light chain stimulates the actin-myosin interaction (29) and the contractile activity responsible for granule centralization (9).

This entire process of actin polymerization and interaction with actin-binding protein and myosin is both blocked and even reversed by cyclic AMP inhibition of the elevation of cytosolic calcium triggered by thrombin or arachidonic acid (10–12). Unlike these activators, Con A precipitates pseudopodal (actin-tropomyosin, alpha-actinin, and actin-binding protein) core components along with most of the glycoprotein II_b-III_a complex in the presence of PGE_1 or dibutyryl cyclic AMP. Under the influence of PGE_1 or dibutyryl cAMP, Con A cannot stimulate phosphorylation of the 20-kD light chain, and the actin-myosin interaction is blocked.

To explain these results, we propose the following model. In the unactivated platelet, it is now generally accepted that ~60–70% of the platelet actin is in a monomeric actin pool (9, 10, 30), perhaps stabilized by profilin (31). The organization and subcellular distribution of the remaining 30–40% actin is not known but has been proposed to be an unorganized or amorphous network (30, 32), possible associated with the platelet membrane (33, 34). This actin may be in short filaments, the ends of which could serve as nucleation sites, and are blocked by capping proteins (35). In our model, the monomeric actin pool is induced to polymerize by receptor occupancy uncapping of these nucleation sites on the membrane or by formation of *de novo* membrane or cytoplasmic nucleation sites. Regulation of this latter process may be accomplished by elevation of cytoplasmic calcium levels causing actin nucleation by gelsolin (36, 37) or, perhaps, by phosphorylation of cytoskeletal proteins such as actin-binding

protein (16, 38). That long actin filaments can form in the presence of gelsolin and micromolar calcium is, perhaps, due to the stabilizing influence of tropomyosin (39), also a component of the platelet cytoskeleton (40).

The cytoskeletal proteins incorporated into the Con A-stimulated cytoskeleton are the same as in the cores recovered from platelets activated with thrombin or arachidonate. However, we suggest that activation may be initiated by an altered mechanism. The binding of Con A to the surface glycoproteins may collect or cap the receptors, resulting in linkage and clustering of polymerized actin (41). This membrane-associated actin could bind alpha-actinin and actin-binding protein on the cytosolic face of the plasma membrane (34). This rearrangement of membrane proteins may also displace calcium from the plasma membrane and initiate phospholipid metabolism resulting in diacylglycerol-mediated protein kinase C activation and cytosolic calcium-mediated myosin kinase activation. This, coupled with actin polymerization triggered by one of the above mechanisms, leads to assembly of the complete cytoskeletal system.

An apparent cytoskeletal core was precipitated by Con A despite cyclic AMP elevation and inhibition of platelet activation in terms of shape change, phosphorylation of 40P and myosin light chain, contractile gel core formation, granule centralization, and secretion. Our interpretation of this result is that we are precipitating membrane-associated complexes of actin-tropomyosin, alpha-actinin, and actin-binding protein already present in the unactivated platelet. A less likely alternative is that these complexes form in the presence of Con A despite cyclic AMP opposition of cytosolic calcium elevation and protein phosphorylation. Although this latter possibility could be analogous to the receptor occupancy-triggered cytoskeletal assembly model mentioned above, we did not observe more than a 5–10% *de novo* increase in filamentous actin in the whole platelet lysates under these conditions. The actin content of the core collected by Con A in the presence of PGE₁ is, most likely, the preexisting pool of 30–40% filamentous actin present in the resting platelet. This would be consistent with a recent finding that a subpopulation of glycoprotein IIb-IIIa is associated with actin in the resting platelet (42).

Actin-binding protein, although phosphorylated by cyclic AMP elevation independent of activation, was not extensively phosphorylated during Con A activation despite almost normal levels of 40P and myosin light chain phosphorylation. Since actin-binding protein was retained in the isolated cytoskeletal core to the same extent as with other activators causing extensive actin-binding protein phosphorylation (9, 16), these results may argue against an obligatory role for such phosphorylation during activation to initiate cytoskeletal interactions. However, it should be noted that the mechanism by which Con A is collecting the actin-binding protein may not be the same as activation-dependent formation of the pseudopodal cytoskeleton. With respect to this possibility, it has been recently reported that actin-binding protein may be associated in the unactivated platelet with glycoprotein Ib (43), a possible component of the Con A cytoskeletal cores.

The behavior of the apparent pseudopodal core from Con A-activated platelets also differs from thrombin- or arachidonate-treated platelets with respect to sensitivity to CB. With these typical activators, CB may inhibit the formation of long actin filaments necessary for pseudopodal formation (9, 10). The cytoskeletal cores contain actin (presumably short fila-

ments) and myosin, but the content of actin-binding protein is substantially reduced. However, in Con A-activated platelets, the extensive Con A cross-linking of the glycoprotein IIb-IIIa complex on the surface allows the actin-binding protein-alpha-actinin-filamentous actin-tropomyosin-membrane complex to be precipitated despite CB pretreatment. The simplest explanation for this result is our previous suggestion (10) that the glycoprotein IIb-IIIa linkage to this cytoskeletal complex remains intact in the presence of CB. While Con A may not organize these complexes into the same pseudopodal cytoskeletal organization, its external cross-linking of these complexes substitutes for the lack of long actin filaments and insufficient cross-linking by actin-binding protein and alpha-actinin.

The contribution of the surface cross-linking to the recovery of glycoprotein IIb-IIIa and actin-binding protein in the core is further illustrated by the increased retention of the actin-binding protein-filamentous actin-glycoprotein IIb-IIIa membrane complex in cytoskeletal cores from platelets activated by thrombin in the presence of the forming fibrin strands despite CB pretreatment. The fibrinogen-fibrin clot provides the equivalent of an external network of multivalent ligands (44). In contrast to Con A multivalent binding, the formation of the glycoprotein IIb-IIIa binding sites for fibrinogen/fibrin is dependent on activation and sensitive to cyclic AMP inhibition (45).

The identity of the cytoskeletal component(s) linking the glycoprotein IIb-IIIa to the cytoskeleton is as yet unknown (46). That extensive cross-linking of the glycoprotein IIb-IIIa complex by an external ligand network, generated by two very different multivalent ligands, retains the actin-binding protein-alpha-actinin-filamentous actin-tropomyosin complex in the cytoskeletal core despite the disruption by CB of the internal cytoskeletal network illustrates the reciprocal nature of these multivalent interactions and the specificity of the glycoprotein IIb-IIIa link to the platelet cytoskeleton.

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