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Protein Changes in Activated Human Platelets

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Using two-dimensional electrophoresis, we mapped both the total and the cytoskeletal proteins of human platelets before and after activation with thrombin or the calcium ionophore A23187. Activation resulted in increased abundance of the phosphorylated form of myosin light chains with an approximate molecular mass of 20 kDa, decreased abundance of two proteins with molecular masses of approximately 18 and 25 kDa, and, in the case of activation with thrombin, the appearance of a new chain of protein spots (named "Thromb:1"). The latter, found associated with isolated detergent-insoluble cytoskeletons, reacted with antibody to human fibrinogen and thus were identified as γ–γ dimers of fibrin. The total number of proteins associated with the cytoskeleton increased after activation with either thrombin or A23187, but we observed some differences in which proteins were bound, and for how long.

Additional Keyphrases: *molecular aspects of platelet morphology and function · electrophoresis, two-dimensional*

Activation of platelets by various substances causes dramatic changes in their morphology and metabolism. Low concentrations of thrombin and collagen cause platelets to change from a discoid to a spherical shape with spiny projections. These activated platelets aggregate, release their granule contents, and cause the retraction of fibrin clots. Many aspects of platelet activation are believed to be mediated by an increase in the intracellular concentration of calcium. In support of this notion, the calcium ionophore A23187 has been found to cause many effects similar to those observed after thrombin activation. On the intracellular level, stimulation of platelets causes phosphorylation of specific proteins via activation of specific kinases [e.g., myosin light chain kinase (*1*), phospholipid-dependent protein kinase (*2*)], reorganization of cytoskeletons (*3*), an increase in actin polymerization (*4*) accompanied by the association of specific proteins with newly formed actin filaments (*3*), and increased contractile activity (*3, 5*).

The phosphorylation of specific platelet proteins after activation appears to be directly related to both changes in cytoskeleton structure and the secretion of granule contents. The two best-described proteins to undergo activation-related phosphorylation are a 57-kDa protein (*6–8*) and a 20-kDa protein (*6, 7, 9*). The former, located in the platelet cytosol, is actually a set of two or three peptides (*8*), which are phosphorylated by a Ca^{2+}-activated, phospholipid-dependent protein kinase (*2*). Haslam et al. (*8*) showed that the phosphorylation of the 47-kDa protein could be uncoupled from aggregation of platelets but is very closely associated with the platelet secretory mechanism (*7*). The 20-kDa protein that is phosphorylated when platelets are activated has been identified as a myosin light chain (*10*). Its phosphorylation is catalyzed by platelet myosin light-chain kinase (*1*), which has been described in both Ca^{2+}-independent and Ca^{2+}-dependent forms (*11*). The phosphorylated form of the myosin light chain has ATPase activity that can be activated by actin (*12*), and the phosphorylated myosin light chain is selectively isolated with detergent-insoluble cytoskeletons prepared from thrombin-activated platelets (*5*). Fox and Phillips (*5*) suggested that, because adenosine diphosphate (ADP) does not cause phosphorylation of myosin light chains under conditions where secretion is inhibited (i.e., no fibrinogen or Ca^{2+}) but phosphorylation does occur along with secretion, the phosphorylation of the 20-kDa myosin light chain and subsequent actin–myosin interaction is a requisite for secretion (*5*).

The increased association of myosin (in the phosphorylated form) with the cytoskeleton of activated platelets is accompanied by an increase in the overall protein content of cytoskeleton preparations (*3, 13, 14*). Using one-dimensional electrophoresis, Phillips et al. demonstrated that the detergent-resistant cytoskeletons of unstimulated platelets contained primarily actin, actin binding protein, a 255-kDa protein, and a small amount of myosin (*13*). Cytoskeletons prepared from thrombin-activated platelets, however, contained higher concentrations of actin, myosin, actin binding protein, and the 225-kDa protein than did the control, plus many other, more minor peptides. Electron microscopy revealed that the cytoskeletons from unstimulated platelets looked like actin filaments, while those from thrombin-activated platelets looked like organized structures that had been extracted intact from individual platelets (*13*). Other than studies on the reversible association of myosin, little attention has been paid to the increased, although transient, association of a seemingly specialized population of proteins with the cytoskeletons during platelet activation.

Fox et al. (*15*) recently published the results of a study in which they used two-dimensional electrophoresis to analyze the protein changes that occur after platelets are activated. Their results included those for proteins, from whole homogenate preparations, with relative molecular masses >40 000. In the present study, we used two-dimensional electrophoresis with an acrylamide gradient in the second dimension that allowed a wider range of molecular mass to be seen, and we examined both total and cytoskeletal proteins from resting and activated human platelets. By using thrombin and the calcium ionophore A23187, we have been able to describe activator-specific protein changes in the platelet protein population and have found that different proteins are associated with the cytoskeleton of activated platelets, depending on the activator used for stimulation. Our two-dimensional electrophoresis patterns also show how much more complex the cytoskeletal protein population of activated platelets is relative to resting platelets, and we provide some additional identification of the proteins associated with the cytoskeleton.

Materials and Methods

Isolation of platelets. For each experiment, 250 mL specimens of human blood were obtained from Hinsdale Hospital after collection during leukophoresis. Acid–citrate–dextrose was used as the anticoagulant during blood collection, and we added two tubes of "Thrombotect" (Abbott Laboratories;

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100 × 16 mm tubes) to each 250-mL specimen before we began to isolate platelets. "Platelet-rich" plasma was prepared by centrifuging the blood at 500 × *g* for 15 min. Platelets were then concentrated from the plasma by centrifugation at 800 × *g* for 20 min. The resulting pellet of platelets was suspended in 20 vol of pH 6.5 buffer containing, per liter, 113 mmol of NaCl, 4.3 mmol of K_2HPO_4, 4.3 mmol of Na_2HPO_4, 24.4 mmol of Na_2HPO_4, 5.5 mmol of glucose, 1.5 mmol of adenosine, and 1 mmol of theophylline (*16*). Preparations contained approximately 10^6 platelets per milliliter, with no detectable leukocytes or erythrocytes.

Activation of platelets. We used 1-mL aliquots of the platelet suspension for activation reactions, initiating activation by adding 0.1 to 0.5 NIH units of thrombin per milliliter or 0.4 μmol of calcium ionophore A23187 per liter. Samples were incubated at room temperature for 0–60 min without stirring, centrifuged for 1 min in a Beckman Model B Microfuge, and the supernates removed.

Preparation of whole homogenates. We dissolved pellets containing activated or non-activated platelets (approximately 0.1-mL packed cell volume) in 0.5 mL of a solution containing, per liter, 9 mol of urea, 40 mL of Nonidet P40 surfactant (NP40), 20 mL of 2-mercaptoethanol, and 100 mL LKB Ampholines (pH 9–11; stock concentration 20% by weight), adjusted to pH 9.5 with NaOH ("NP40–urea mix"). We used 10 to 20 μL of each sample for two-dimensional electrophoresis gels, which we stained with Coomassie Brilliant Blue. For gels that were to be stained with silver, each sample was diluted with an equal volume of the same NP40–urea mixture and 5–10 μL was used per gel.

Preparation of cytoskeletons. We prepared cytoskeletons from control and activated platelets by resuspending platelet pellets in 1 mL of buffer containing, per liter: 5 mL of Nonidet P40, 0.1 mol of piperazine-*N*,*N*′-bis[2-ethane sulfonic acid], 2 mmol of ethylene-glycol-bis[β-amino-ethyl-ether)-*N*,*N*′-tetraacetic acid (EGTA), 1 mmol of $MgSO_4$, 1 mmol of phenylmethyl sulfonyl fluoride, 1 mmol of pepstatin A, and 2 mol of glycerol, adjusted to pH 6.9 (*17*). After resuspension was complete, an additional 9 mL of the buffer was added. The detergent-resistant cytoskeleton material was collected by centrifugation for 1 h at 70 000 × *g*. Cytoskeleton pellets were dissolved in 0.25 mL of NP40–urea, and 10 to 20 μL of each sample was subjected to two-dimensional electrophoresis, and the gels were stained with Coomassie Brilliant Blue (5–10 μL of samples diluted 1:1 with additional NP40–urea was used on gels to be stained with silver).

Two-dimensional electrophoresis. First-dimension isoelectric focusing was done as previously described (*18*) with LKB ampholytes (75 μL of pH 2.5–4 plus 675 μL of pH 3.5–9.5). After equilibration with sodium dodecyl sulfate (SDS) (*19*), proteins were separated in the second-dimension by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels that were linear gradients of polyacrylamide, 90–280 g/L. Details of the SDS-PAGE procedures were as previously described (*20*). Slab gels were fixed and stained in Coomassie Blue solution as before (*21*) or were fixed for at least 4 h in a solution containing, per liter, 500 mL of ethanol with 1 mL of formaldehyde and stained with silver according to the method of Guevara et al. (*22*), with use of half the amount of citric acid and formaldehyde described in that paper for development.

Immunochemical staining. Proteins with homogenates of human platelets that were thrombin-activated for 10 min were transferred from polyacrylamide gels to nitrocellulose and free binding sites on the nitrocellulose were blocked with a 30 g/L solution of bovine serum albumin as described by Anderson et al. (*23*). The nitrocellulose transfers were then reacted with rabbit antihuman fibrinogen (Miles Laboratories, Inc., Elkhart, IN 46515) or mouse antichicken α-tubulin (Amersham, Arlington Heights, IL 60005). Proteins that bound these antibodies were detected by incubating the nitrocellulose transfers with an appropriate peroxidase-conjugated anti-immunoglobulin (Miles Laboratories) such as antirabbit IgG and subsequently reacting the transfers with horseradish peroxidase reagent (BioRad Labs., Richmond, CA 94804) and hydrogen peroxide.

Protein identification. We identified the myosin heavy chains, actin, and α-actinin by co-migration with a homogenate of human skeletal muscle in which the positions of the major contractile and structural proteins have been previously identified (*24*). Gelsolin was identified by co-migration with purified gelsolin (a gift from Dr. K. Okuda, Osaka City Medical School, Osaka, Japan) and also by comparison with the published identification of Fox et al. (*15*). The platelet tropomyosins were identified by their co-purification in nonmuscle tropomyosin preparations done according to Fine et al. (*25*), their shift to a higher apparent relative molecular mass when the second dimension separation was done in the presence of urea (*26*), and their reactivity with antibodies to tropomyosin (*27*). Myosin light chains were identified by their migration pattern, which was similar to that of myosin preparations from human fibroblasts (unpublished data). Tubulin and the subunits of fibrinogen were identified by staining nitrocellulose transfers to two-dimensional gels with specific antisera (*23*). Albumin was identified by comigration with human plasma (*28*).

Results

Figure 1 shows two-dimensional electrophoretic patterns of total and cytoskeletal proteins from platelets before and after treatment with 0.5 NIH units of thrombin per milliliter of incubation medium. Patterns identical to that shown in Panels *D* or *H* were obtained after 20-, 30-, and 60-min incubations. An increase in the acidic (phosphorylated) form of the myosin light chains was observed after only a 1-min incubation with thrombin. This transition was reversible, with the acidic forms decreasing to almost control levels by 20 min. Two unidentified proteins (indicated in Figure 1 with a vertical arrow, pointing down) with approximate relative molecular masses of 18 000 and 25 000 decreased progressively when platelets were incubated with thrombin; only a trace amount remained after 10–20 min. A chain of polypeptides (Thromb:1) appeared in the patterns after approximately 5 min of incubation with thrombin (the time of appearance varied from 5 to 30 min, according to the batch of thrombin used) and increased in abundance up to 60 min, the longest interval we studied. Thromb:1 migrates to the same approximate position as α-actinin in the two-dimensional gel system used; therefore it has an SDS-molecular mass of approximately 100 000 (*30*).

The cytoskeleton preparations from control platelets contained a few major proteins—including actin, tubulin, α-actinin, and tropomyosin—plus a small population of unidentified proteins, present in low abundance, and a trace amount of the myosin light chains. After a short incubation with thrombin, however, a more complex pattern of proteins associated with the cytoskeleton appeared (Figure 1*F*). The myosin light chains, fibrinogen subunits, gelsolin, and several poorly resolved, high-M_r proteins (large arrows pointing up in Figure 1, *F–H*) were more prominent than in the control. In addition, several proteins not seen at all in the control cytoskeletons were associated with the platelet cytoskeleton after brief exposure to thrombin. These included the 25-kDa protein described above in the total platelet-protein analysis, which decreases in abundance after throm-

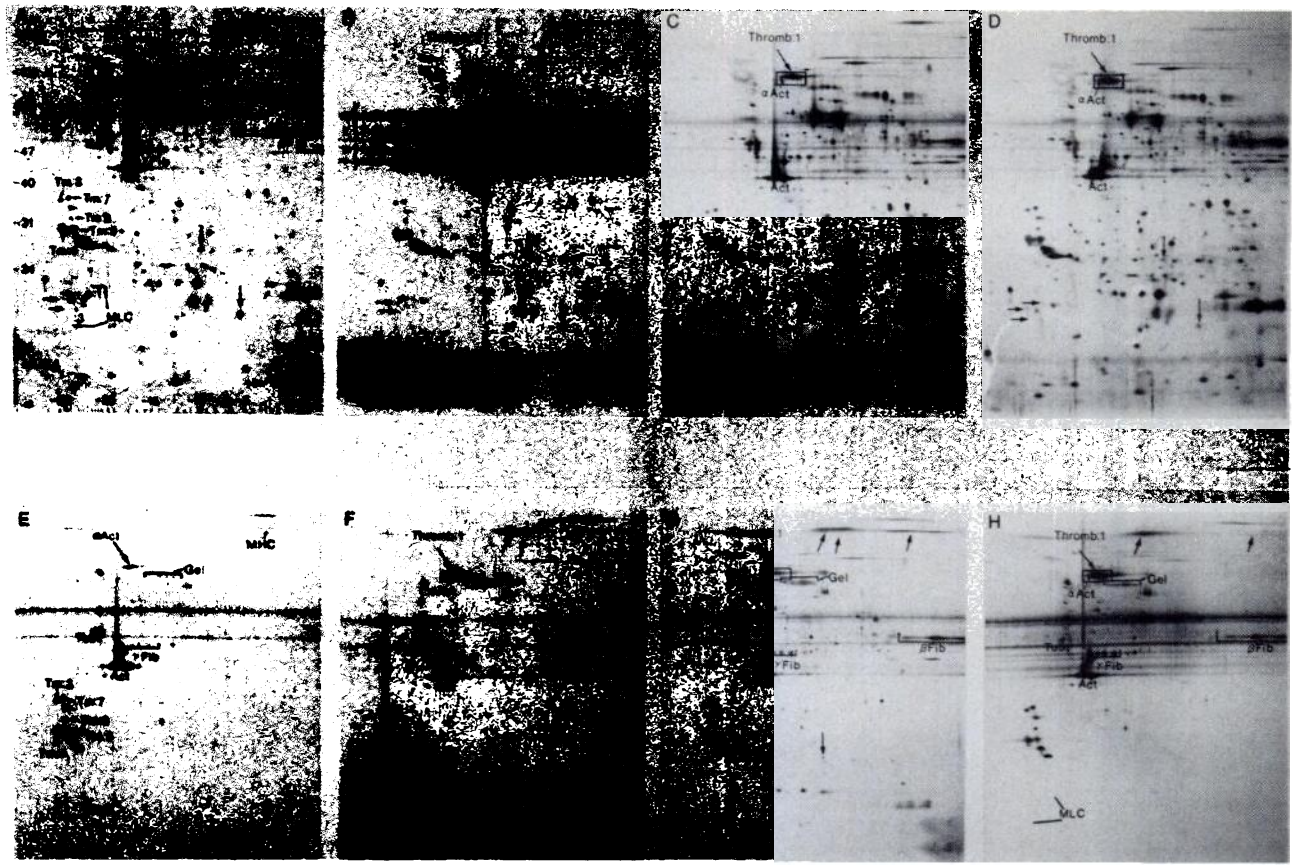

Fig. 1. Two-dimensional electrophoresis patterns of total proteins (*A–D*) and cytoskeletal proteins (*E–H*) from resting and thrombin-activated platelets

Platelets were isolated and activated with 0.5 NIH units of thrombin per milliliter of incubation medium. Homogenates of total platelet protein were prepared as described from (*A*) control platelets and platelets that had been activated for (*B*) 1, (*C*) 5, and (*D*) 10 min. Cytoskeletons were prepared as described from (*E*) control platelets and platelets that had been activated for (*F*) 1, (*G*) 5, and (*H*) 10 min. *MHC*, myosin heavy chains; *α-Act*, α-actinin; *Gel*, gelsolin; *Alb*, albumin; *α-*, *β-*, and *γ-Fib*, fibrinogen chains; *Tub*, tubulin; *Act*, actin; *Tm*, tropomyosin; *MLC*, myosin light chains; *Thromb:1*, proteins that appear after activation with thrombin. *Horizontal arrows* indicate phosphorylated forms of myosin light chains; *vertical down-pointing arrows* indicate proteins that decrease in abundance after activation; *vertical up-pointing arrows* show unidentified proteins that increase in abundance in cytoskeletons after activation. Molecular-mass scale on *y*-axis of *A* was obtained by using plasma proteins as an internal standard (*28*); charge units on *x*-axis of *A* were determined by using carbamylated creatine kinase (EC 2.7.3.2) as an internal standard (*29*). Patterns are oriented with the acidic side to the left and basic side to the right

bin activation. Most of these proteins, including the myosin light chains, were only transiently associated with the cytoskeleton. However, a few, including Thromb:1, the fibrinogen subunits, and gelsolin, remained associated at concentrations exceeding the control after 20 min of incubation with thrombin. The amount of α-actinin associated with the cytoskeleton appeared to remain the same throughout the period of thrombin incubation studied.

Activation of platelets with the calcium ionophore A23187 resulted in some of the same changes in the protein patterns found after incubation with thrombin (Figure 2; patterns similar to those shown in panels *D* and *H* were obtained after 5-, 10-, and 20-min incubations). A quantitative increase in the phosphorylated form of the myosin light chains was obvious just 1 min after A23187 was added (Figure 2*B*). The abundance of the 18- and 25-kDa proteins decreased, also by 1 min. Thromb:1 did not appear, however, even after incubation for 60 min. As in the case of thrombin activation, the increased abundance of the phosphorylated myosin light chains was transient, with a return to control levels by 30 min after the A23187 was added. The abundance of the 18- and 25-kDa proteins was unchanged, well below that of the control, after 30 min of incubation with A23187 (Figure 2*C*) and at 60 minutes (Figure 2*D*).

As with platelets activated by thrombin, more proteins were found associated with the cytoskeletons prepared from platelets incubated with A23187 for 1 min than with those prepared from control platelets (Figure 2, *E–F*). The proteins more abundant in cytoskeletons of A23187-treated platelets included α-actinin, gelsolin, the myosin light chains, several poorly resolved high-M_r proteins that are as yet unidentified (large arrows in Figure 2*F*), and the 25-kDa protein that showed a decreased abundance in the whole-protein pattern. Unlike the case of thrombin-activated platelets, in the A23187-activated platelets the association of *all* of these proteins was transient (Figure 2, *G–H*). Thirty minutes after the ionophore was added (Figure 2*G*) the cytoskeleton protein pattern was identical to that of the control. Also in contrast to the thrombin-activated platelets, the A23187-activated platelets showed no increased association of the fibrinogen subunits with the cytoskeleton, even after 60 min of incubation with the ionophore.

Activation of platelets with ADP (0.1 μmol per liter of incubation medium) and collagen (20 μg per milliliter of incubation medium) also failed to stimulate the appearance of Thromb:1 (data not shown), although the increase in the phosphorylated form of the myosin light chains and the decrease in the 18- and 25-kDa protein were observed.

Enlargement of the Thromb:1 region from Figure 1, *A–D*, shows the charge and molecular-mass heterogeneity of this protein set (Figure 3). When a nitrocellulose transfer of the protein pattern shown in Figure 1*D* was "stained" by use of antihuman fibrinogen, spots corresponding to γ−γ dimer and α-, β-, and γ-fibrinogen chains were detected. In addi-

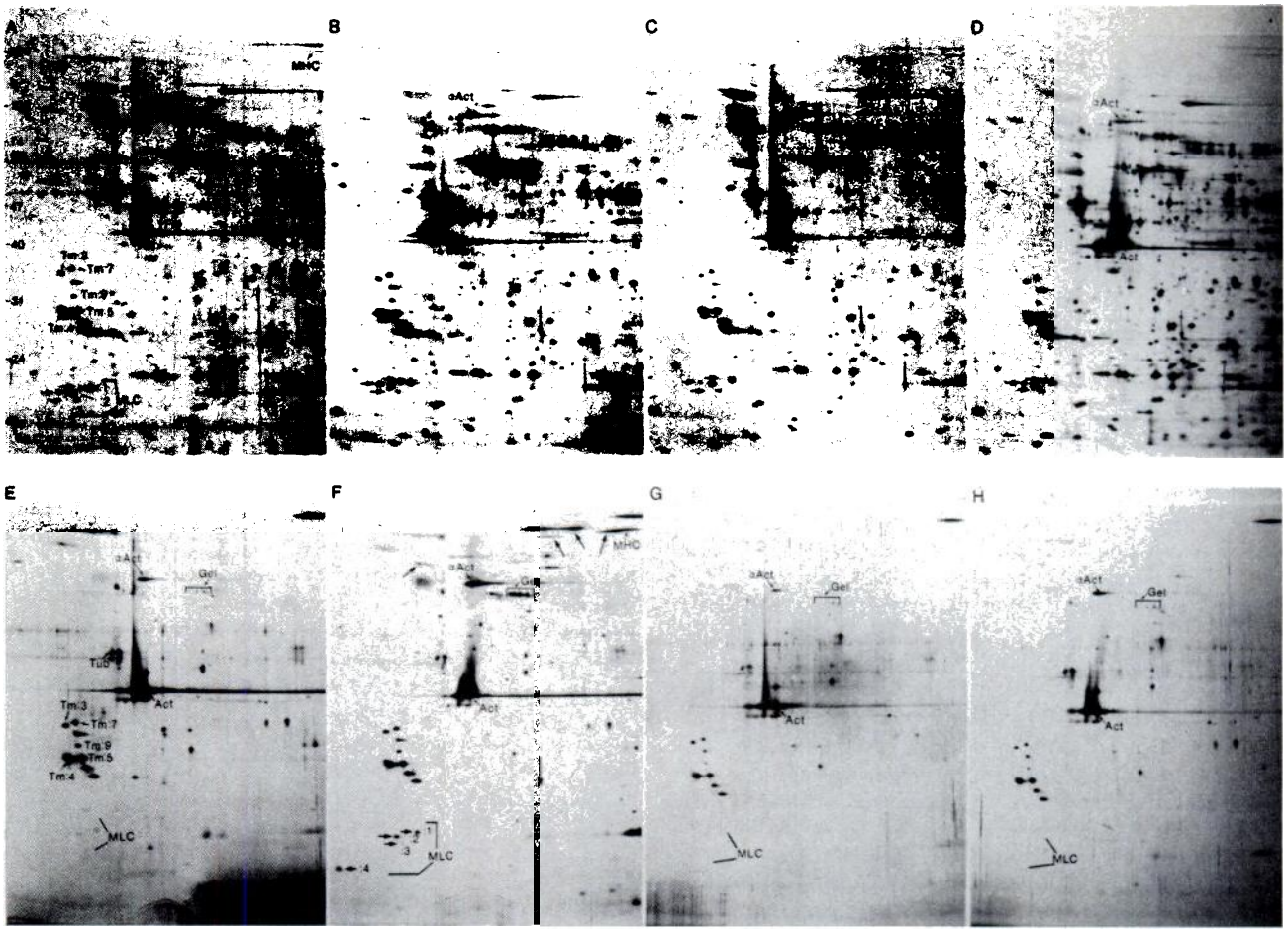

Fig. 2. Two-dimensional electrophoresis patterns of total and cytoskeletal proteins from resting and A23187-activated platelets

Platelets were activated with 0.4 μmol of A23187 per liter and homogenates of total platelet protein were prepared from (*A*) control platelets and platelets activated for (*B*) 1, (*C*) 30, and (*D*) 60 min. Cytoskeletons were prepared from (*E*) control platelets and platelets activated for (*F*) 1, (*G*) 30, and (*H*) 60 min. Abbreviations, standardization, symbols, and gel orientations are all as described for Figure 1

tion to the α-, β-, and γ-subunits of fibrinogen—the identification of which is based on the positions of these peptides in two-dimensional electrophoresis patterns of human plasma (*28*)—a chain of high-M_r spots corresponding to Thromb:1 was stained.

Discussion

Two-dimensional electrophoretic patterns of total proteins from platelets activated with either thrombin or A23187 differed in three ways from patterns for unactivated platelets: (*a*) new protein spots appeared (e.g., Thromb:1), (*b*) some proteins increased in abundance (e.g., the phosphorylated form of myosin light chains), and (*c*) some decreased in abundance (e.g., the 18- and 25-kDa proteins). Except for Thromb:1, which appeared only when thrombin was used, thrombin and A23187 caused the same changes in the total protein pattern. The increased abundance of the phosphorylated form of the myosin light chains we observed is in agreement with results of other investigators, who used one-dimensional electrophoresis (*5*). The decreased abundance of the 18- and 25-kDa proteins, previously unreported, could be due to activation of a specific protease during the platelet activation process or to the secretion of those particular proteins from the platelets as part of the secretion process associated with platelet activation.

Phillips et al. (*13*) reported previously that the amount of detergent-insoluble material increased when platelets were activated before extraction, and others have confirmed this observation (*3, 14*). Using two-dimensional electrophoresis to resolve the proteins, we also found that the number of proteins associated with the cytoskeleton was greater for platelets activated with thrombin or A23187 than for control platelets. Because staining with silver is approximately 100 times more sensitive than staining with Coomassie Blue for detection of protein in polyacrylamide gels (*31*), we have been able to show more proteins, even in the control platelet preparations, than have been described previously (*3, 13, 14*). Unlike the platelet cytoskeleton preparations described by Rosenberg et al. (*32*), our preparations con-

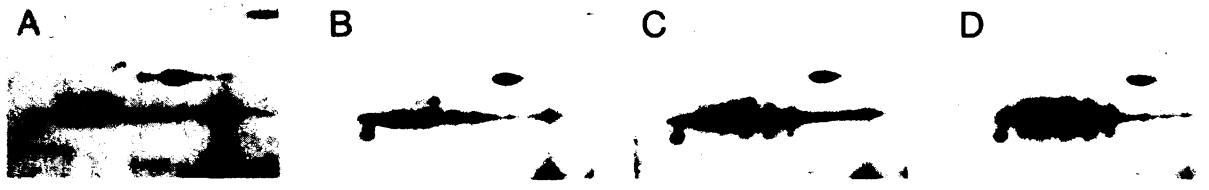

Fig. 3. The photographic enlargement of Thromb:1 region from Figure 1, *A–D*, shows the charge and molecular-mass heterogeneity of this set of polypeptides

tained substantial amounts of tubulin—a preservation probably ascribable to our including glycerol in the cytoskeleton preparation buffer to stabilize microtubules (*33*) and to the fact that we isolated the cytoskeletons at room temperature rather than on ice [microtubules dissociate in the cold (*33*)]. In addition to tubulin, our control platelets contained tropomyosin, actin, α-actinin, a small amount of gelsolin and myosin (both heavy and light chains), and approximately 50 other unidentified proteins in various abundances.

Activation by either thrombin or A23187 caused an increase in the number of proteins associated with the cytoskeleton, but there were differences in the specific proteins that became associated and differences in the duration of their association. The amount of fibrinogen associated with the cytoskeletons was increased by thrombin but not A23187, and Thromb:1 appeared only after activation with thrombin.

Fox et al. (*15*) also reported the appearance of a protein with a molecular mass similar to Thromb:1 (peptide 4, ref. *15*) after activation with thrombin, and suggested that it was the γ–γ dimer of fibrin. Our data showing the specific reactivity of Thromb:1 with antibody to fibrinogen confirms that suggestion. The association of the fibrinogen subunits with the cytoskeleton and the fact that Thromb:1 did not appear after activation with A23187, ADP, or collagen suggest that thrombin causes the specific binding of fibrinogen to the platelet cytoskeleton and subsequent conversion to fibrin. There is evidence that fibrinogen is present both in surface-associated form and intracellularly in platelets, and that the intracellular fibrinogen is released from α-granules after platelet activation as part of the secretory response (*34*). McDonagh et al. (*35*) found that platelets release sufficient Factor XIII after activation (e.g., ADP plus fibrinogen) to cause formation of γ–γ dimers of fibrin. It remains to be elucidated whether these dimers are on the outer surface of the activated platelets and appear in the cytoskeletal protein patterns as a result of membrane contamination in the cytoskeleton preparations or are formed by cross-linking of intracellular fibrinogen subunits by released Factor XIII and thus associate with the filament structure of the cytoskeleton. Previous reports suggest that the membrane contamination in platelet cytoskeletons is low, although cytoskeletons from aggregated platelets may contain detectable amounts of membrane glycoproteins IIb and III (*13*). We are now investigating the localization of the fibrinogen found in our platelet preparations by analysis of platelet membrane preparations and use of platelets surface-labeled with ^{125}I for cytoskeleton preparations.

Fox et al. (*15*) reported a group of three high-M_r proteins (peptides 1–3) that appeared in two-dimensional electrophoresis patterns after incubation of platelets with thrombin, collagen, or A23187. These proteins were not generated if EGTA or inhibitors of calcium-dependent proteases such as leupeptin were included in the reaction mixture, which led them to suggest that these proteins were the products of the calcium-dependent protease activated during platelet aggregation. We failed to find any of the three peptides described by Fox et al., although we did find and resolve more clearly a protein comparable to their peptide 4. Fox et al. stirred their preparations during incubation; we did not, but an experiment comparing the results after agitating or not agitating the platelets showed no difference in our platelet protein patterns (data not shown). Fox et al. also reported that peptides 1–3 did not appear if EDTA (an inhibitor of platelet aggregation) was included in the incubation buffer. We had visible aggregation in both the thrombin and A23187 preparations after various incubation intervals (10–30 min for thrombin; 0.5–5 min for A23187). The only obvious difference between our procedures and those of Fox et al. is our use of a buffer that contained low concentrations of theophylline (1 mmol/L) and adenosine (1.5 mmol/L) for the activation reaction. The theophylline keeps platelets from being "spontaneously" stimulated before thrombin or A23187 is added. Perhaps this alteration of conditions caused the Ca^{2+}-dependent protease to remain inactive.

A notable difference between the activation of platelets with thrombin and A23187 is the longevity of the association of specific proteins with the cytoskeleton. Cytoskeletons prepared from platelets that were activated with thrombin showed a constant level of associated α-actinin, comparable to the control, throughout the incubation period studied. Cytoskeletons prepared from platelets activated with A23187 for 1 min, however, showed a definite increase in the amount of associated α-actinin, with a decrease to control levels after longer periods of incubation. Rosenberg et al. (*32*) reported that the association of α-actinin with the platelet cytoskeleton is Ca^{2+}-dependent. Therefore the obvious increase in α-actinin association with the cytoskeleton after treatment with A23187 could be a result of an sudden increase in cytosol Ca^{2+} concentration owing to the activity of the calcium ionophore. Thrombin activation also alters the intracellular Ca^{2+} concentration, but probably through interaction with membrane receptors and a second-messenger mechanism rather than directly as with A23187.

We observed an increase in the phosphorylated form of three myosin light chain proteins with molecular masses of approximately 20 kDa (approximate difference among the three is 500 Da) in the two-dimensional electrophoretic patterns of total proteins from platelets activated for just 1 min with either thrombin or A23187. Previous investigators have reported that platelet myosin contains two 20-kDa light chains (*36*) and that these light chains can be phosphorylated (*10*). Pollard et al. (*36*), however, showed a small peak that eluted between the 20-kDa and 15-kDa myosin light chains on gel filtration, suggesting the possibility of an additional myosin subunit. Therefore, our observation of three myosin light chains with molecular masses all near 20 kDa is most probably attributable to the superior resolution of our two-dimensional gel system.

As reported by others using one-dimensional electrophoresis techniques (*5*), the phosphorylation of the myosin light chains was directly correlated with the increased association of the myosin (both heavy and light chains) with the cytoskeleton. The myosin light chains were partly dephosphorylated, however, during our cytoskeleton preparation procedure, as shown by the decreased abundance of the more-acidic forms in the cytoskeleton patterns as compared with the total protein patterns of proteins isolated after one minute of activation (Figure 1, *B* and *F;* Figure 2, *B* and *F*). Inclusion of sodium fluoride to inhibit phosphatase activity protected the myosin light chains from dephosphorylation, but decreased the amount of myosin associated with the cytoskeleton as well (data not shown). A similar decrease in association of myosin was observed by Fox and Phillips (*5*), who used a combination of potassium phosphate, sodium pyrophosphate, and sodium molybdate as phosphatase inhibitors. Our purpose was to evaluate the proteins associated with the cytoskeleton after activation of platelets, so we decided to eliminate phosphatase inhibitors from the preparation buffer in order to obtain more nearly complete cytoskeletal protein populations at the expense of preserving the phosphorylated form of the myosin light chains.

We did not find the P47 protein, which is reportedly phosphorylated in platelets after activation with thrombin and is thought to play a role in secretion of platelet granules (*6, 8*). The obvious phosphorylation of the myosin light

chains in our study serves as a control, showing that the expected effects of activation did occur. P47 has a pI between 6.0 and 6.5 (*8*) and an approximate M_r of 40–47 kDa (*2, 6, 8, 14*), depending on the SDS electrophoresis system used for analysis. Therefore this protein may not have been resolved from actin in our patterns. Imaoko et al. (*8*) showed that P47 stains very faintly relative to actin with Coomassie Brilliant Blue, and therefore would be easily overpowered by the high concentration of actin in the preparations we analyzed.

This study has shown that different substances that activate platelets can cause some specific differences in the proteins that are associated with the cytoskeleton, and in the duration of those protein associations. More studies are needed to test the effects of other activators such as ADP, collagen, and ristocetin, in order to unravel the various mechanisms of these different effects and perhaps to provide information on the identity of the proteins that become associated transiently with the cytoskeleton. This report represents the beginning of a data base on normal platelet proteins that will continue to grow as more studies are done. The long-term objectives are to explain protein alterations due to diseases affecting platelets in the context of the normal protein population and to describe the molecular events occurring after activation or treatment with drugs.

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