

# Platelet Activation and Microfilament Bundling

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**ABSTRACT** Human platelets were obtained in the fully resting state by treating discoid populations with 1.5 mM tetracaine and in the activated state by treatment with 2  $\mu$ M A-23187. After gel filtration or washing, respectively, platelet suspensions were lysed with 1% Triton X-100 at pH 6.8. The precipitates from resting platelets viewed by negative staining appeared predominantly granular with a few very short microfilaments. They contained polypeptides of 250, 100, 45, 38, 36.5, and 35 Kdaltons, and three small polypeptides including one with the mobility of profilin on SDS gels. Precipitates from activated platelets lacked this low molecular weight band and contained a major band at 200 Kdaltons with the mobility of myosin; these precipitates had significant  $K^+$ ,  $Ca^{++}$  ATPase activity absent from the precipitate of resting platelets. As seen in negative staining, precipitates from activated platelets contained microfilaments arranged as nets or bundles. The granular resting precipitates were transformed in vitro into microfilament bundles by washing the precipitates in buffer at higher pH (7.6) in the presence of  $5 \times 10^{-5}$  M calcium chloride.

Blood platelets change shape in seconds from disks to small spheres with many fine filopodia when they are activated by various physiological agonists or by the ionophore A-23187. During this event they also develop the capacity to aggregate, which was previously suppressed. This remarkable change therefore has general implications for the mechanism of formation of filopodia by cells and also for surface alterations that can lead to thrombi in vivo.

Recently, it was shown by examination of single discoid platelets which were rapidly lysed on electron microscope grids and negatively stained that the resting ground cytoplasm remaining on the grid was granular in character. After shape change was induced, this amorphous-appearing material was replaced by nets of microfilaments in the platelet body and bundles of actin containing microfilaments in the filopodia (16, 17).

Here we show that when suspensions of whole populations of resting and activated platelets are rapidly lysed following the procedure of Lucas et al. (13), the precipitates that form give independent evidence that this replacement reflects a transformation. The granular precipitate formed from resting platelets can be converted in vitro into remarkable bundles of microfilaments that in some cases closely resemble filopodia. We propose that this conversion represents the first in vitro model of platelet shape change.

## MATERIALS AND METHODS

### *Preparations of Platelets*

Approximately 100 ml of blood (after discarding the first 1 ml) was allowed to flow freely through a No. 19 needle from the antecubital vein of a healthy donor into a solution of 2.5% sodium citrate, 1.5% citric acid, and 2% dextrose ( $\frac{1}{10}$  vol). The blood was mixed by inversion and immediately centrifuged at 250 g, 37°C, for 10 min to remove erythrocytes and leukocytes. The platelet-rich plasma (PRP) was examined to determine the percentage of discoid (resting) platelets. All procedures were carried out with plastic tubes or siliconized glass-

ware. Platelet counts were done with a Zeiss phase contrast microscope at  $\times 1,250$  using an objective of NA 1.30. The proportion of discoid forms and filopodia could be easily detected. A platelet count of  $>80\%$  discoid with few filopodia was considered a good "resting" population and was further processed. The PRP was divided into two equal aliquots. To one, tetracaine was added from a 100-mM solution in 50% methyl sulfoxide to a final concentration of 1.5 mM tetracaine. EGTA was also added to a final concentration of 10 mM. Platelets were treated with the EGTA-tetracaine mixture for 5 min at 37°C, re-examined, and then separated from the plasma by rapid gel filtration through Sepharose 2B at 37°C in plastic columns. The eluant was 0.1 M KCl, 7 mM imidazole, 10 mM EGTA (buffer A) at pH 6.8 with the addition of 1.5 mM tetracaine. After gel filtration, platelets were re-examined to determine the persistence of discoid forms.

Activated platelets were obtained by bringing the second aliquot of PRP to a final concentration of 10 mM EGTA, 2  $\mu$ M A-23187. After a few minutes the percentage of activated forms was determined; it was usually  $>80\%$ . Activated platelets were small spheres with many filopodia. Platelets were spun out of the PRP at 1,000 g for 15 min and washed twice in buffer A. They were resuspended in 2-3 ml of buffer A at pH 6.8 and re-examined to determine the persistence of activated forms.

### *SDS Gel Analysis*

Suspensions of resting and activated platelets were lysed rapidly essentially by the method of Lucas et al. (13) by adding 10% Triton X-100 containing phenylmethylsulfonyl fluoride (PMSF) and leupeptin to achieve a final concentration of 1% detergent with  $10^{-4}$  M PMSF and 0.1% leupeptin. The samples were immediately placed on ice. After 5 min a precipitate developed, and the samples were then spun for 15 min at 1,000 g at 4°C. Precipitates were washed three times at 4°C with buffer A, pH 6.8. The precipitates and the initial supernates were prepared for SDS gel electrophoresis by the method of Laemmli (11), with the modifications that the procedure was adapted for microslab gels as described by Matsudaira and Burgess (15), and the acrylamide in the stacking gel was increased to 4.75%. Electrophoresis was carried out at a constant voltage of 150 V. Gels were stained in 0.25% Coomassie Brilliant Blue R in 50% methanol, 10% acetic acid, and destained in 5% methanol, 10% acetic acid. Tests for calcium shift were carried out by adding to a well twice the amount of calcium as EGTA present in the sample, so as to bring the free calcium to 5 mM (10).

### *Negative Staining*

Washed precipitates were resuspended in 1-2 ml of buffer and were negatively stained with 1% uranyl acetate or with 2% ammonium molybdate in 10 mM

imidazole, pH 7.0. Carbon-coated grids were examined in a Philips 201 electron microscope at 80 kV using a platinum aperture of 50  $\mu\text{m}$  diameter. Frequently, grids subjected to glow discharge were used or grids were rinsed with cytochrome *c* as previously described (16).  $\text{K}^+$ ,  $\text{Ca}^{++}$  ATPase activity was determined by the method of Adelman and Taylor (1). Heavy meromyosin subfragment was added to grids at 0.1–0.2 mg/ml before rinsing and staining.

Tetracaine was obtained from Pfaltz & Bauer Inc. (Stamford, Conn.). Dimethyl sulfoxide was a product of Fisher Scientific Co. (Pittsburgh, Pa.).

## RESULTS

### *Platelet Populations Compared*

Platelets found to be >80% discoid in plasma were treated with 1.5 mM tetracaine in 0.8% dimethyl sulfoxide for 5 min to inhibit shape change as previously described (18). As shown in the previous scanning electron micrographs, the platelets retract filopodia and appear slightly rounded from the disk shape (18) but are not swollen as in lidocaine (17). A second aliquot of

platelets was activated as described with the ionophore A-23187 to induce shape change to small spheres with long filopodia. The respective morphologies were well retained through either rapid gel filtration (tetracaine) or two rapid washes (ionophore); it is estimated that up to 10% of the platelets may swell during these procedures, and rapid treatments are necessary to keep this to a minimum. Gel filtration of activated platelets cannot be used, as it results in aggregation and retention on the column.

### *Character of the Precipitates*

When the tetracaine gel-filtered platelets were rapidly lysed with Triton X-100 as described, and when the precipitate was collected, diluted in buffer A, and examined after negative staining, the precipitate was found to be predominantly amorphous or granular in character as shown in Fig. 1. Note that

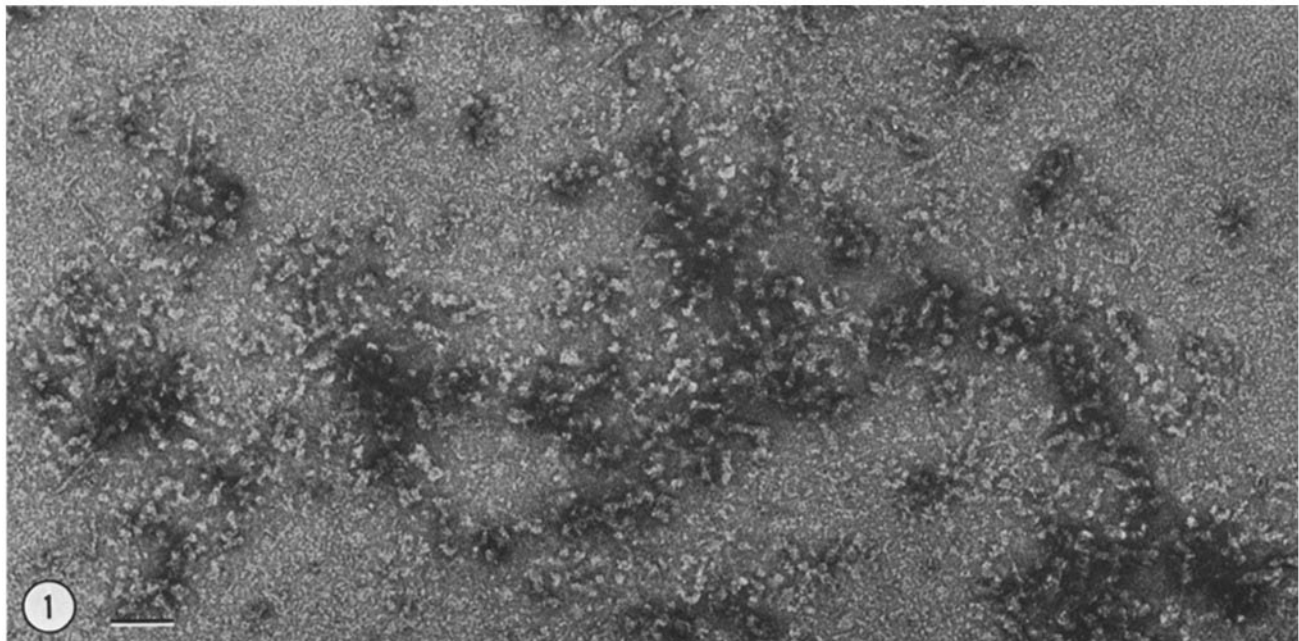


FIGURE 1 Complete amorphous precipitate from a platelet population that was >80% discoid before treatment with 1.5 mM tetracaine. Note single short microfilament at top.  $\times 81,200$ .

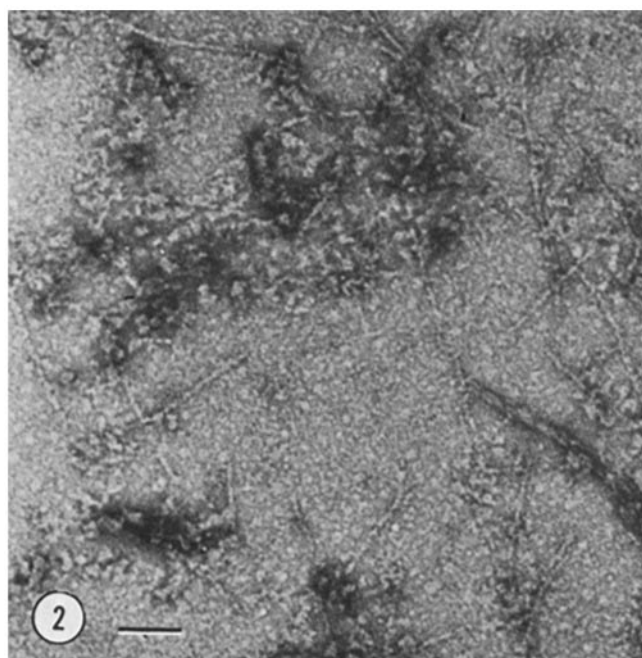


FIGURE 2 Precipitate consisting of both filaments and amorphous aggregates. From a partially activated platelet population.  $\times 84,000$ . All figures are samples of the precipitates negatively stained with uranyl acetate. The bar on each one represents 0.1  $\mu\text{m}$ .

the few microfilaments are  $<0.1 \mu\text{m}$  in length; this was the case in our best preparations. We noticed that the anesthetic caused considerable filopodial retraction even in partly activated populations, but precipitates from these preparations contained both amorphous materials and many longer filaments as previously described (13). An example from such a preparation is shown in Fig. 2.

In contrast to both these types of precipitates, those from the ionophore-treated platelets contained some free or loosely connected filaments and also a varying number of microfilament bundles. Fig. 3 is an example.

The individual subunits of the amorphous aggregates of Fig. 1 measure 12–25 nm in diameter. They are rarely found free but tend to stick together, often forming S- or L-shaped aggregates. The free filaments and filaments in bundles found in Figs. 2 and 3 measure 4 nm in diameter.

### *In Vitro Conversion of the Amorphous Precipitates*

We discovered that the amorphous precipitate could be transformed into microfilament bundles by resuspending it for 15 min or longer in 0.1 M KCl, 7 mM imidazole at pH 7.6, with  $5 \times 10^{-5}$  M  $\text{CaCl}_2$ . Examples of bundles are shown in Figs. 4, 5, 7, and 8.

It was estimated that about one-third to one-half of the precipitate forms bundles, many of them small but, in several areas, aggregating into larger bundles. Small amorphous aggregates remained in other areas of the grids.

Fig. 4 shows an example of a negatively stained region showing bundles of up to  $\sim 10$  microfilaments together with more loosely arranged nets of microfilaments.

Fig. 5 shows the results of the addition of heavy meromyosin subfragment one to the transformed precipitate before negative staining. Microfilaments are decorated, and in the more isolated bundle (arrow) all the filaments that can be resolved (9 out of 11) have polarities in the indicated direction. Two could not be scored. Figs. 7 and 8 are examples of individual transformed bundles which appear similar to the bundle in Fig. 6 from an ionophore precipitate.

We tested the effect of pH and calcium separately on the precipitate. The amorphous precipitate was resuspended in the presence of  $5 \times 10^{-5}$  M calcium at pH 6.8, or without divalent

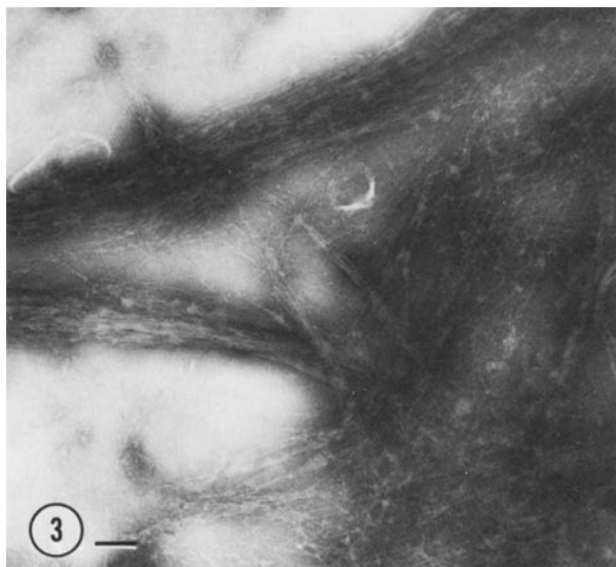


FIGURE 3 Nets and bundles of filaments from an A-23187-activated platelet population. 2% ammonium molybdate.  $\times 54,000$ .

ions (buffer A) at pH 6.8 or 7.6. Transformation to the crystalline bundles occurred only in the presence of calcium at pH 7.6. Little or no bundling was observed under the other conditions.

### *Polypeptide Components of the Precipitates*

When the precipitates were solubilized in SDS and subjected to gel electrophoresis, we observed several differences in the components of the precipitates from resting and activated platelets. The precipitates from the tetracaine-treated platelets contained polypeptides of 250, 100, and 42 Kdaltons (Fig. 9), and several lower molecular weight polypeptides estimated as 38, 36.5, 35, 21, 19.8, and one estimated as 16.5 Kdaltons, running just above the hemoglobin standard (Fig. 10). The molecular weights were determined on a semi-log plot using several standards. The 100-Kdalton polypeptide was found to comigrate with alpha-actinin (Fig. 9, lane 5). There was no band visible at 200 Kdaltons in precipitates from fully resting platelets (Fig. 9, lane 3). Trace amounts of other polypeptides were seen. In quite striking contrast, the precipitate from ionophore-treated platelets contained polypeptides at both 250 and 230 Kdaltons, a major band at 200 Kdaltons and a band at 135 Kdaltons, as well as the 100- and 42-Kdalton bands, and several other polypeptides present in small amounts (Fig. 9, lane 4). The low molecular weight bands seen in the resting platelet precipitates were reduced or absent, and there was no band at 16.5 Kdaltons (Fig. 10, lane 4). The intensity of the band at 200 Kdaltons appeared too great to be accounted for by proteolysis of the 250- and 230-Kdalton bands. We tested both precipitates for  $\text{K}^+$ ,  $\text{Ca}^{++}$  ATPase activity as described. We found that the "ionophore" precipitate exhibited such activity while it was absent from resting precipitates (see Table 1). Comparison of the supernates showed a relative depletion of the 200-Kdalton peak in the supernate from activated cells as shown by comparing lanes 6 and 7 in Fig. 9. Another difference between the precipitates was the presence in the activated one of minor polypeptides at 95, 80, 74, 58, 53, and 48 Kdaltons (lane 4) which are depleted or absent in the activated supernate (lane 7) but present in the supernate from tetracaine-treated cells (lane 6).

To see whether there was a loss in any of the components from the resting precipitate after transformation in vitro, we washed transformed precipitates three times and then subjected them to electrophoresis. A slight depletion in the 100-Kdalton polypeptide was consistently observed as shown in lane 9 of Fig. 9 (compare with lane 3) and in lane 8 of Fig. 10 (compare with lane 3). There also appears to be some depletion in the third band of the triplet below actin.

To test whether either component was capable of altering mobility on SDS gels in the presence of calcium, we ran precipitates from resting platelets in the presence of EGTA or 5 mM calcium under conditions used for calmodulin (8, 10). No shift in either component was observed.

### DISCUSSION

We conclude from these observations and from our previous observations of rapidly lysed single platelets (16, 17) that resting platelets contain a precursor of filamentous actin which is either complexed with several components in the platelet or rapidly complexes with them to form the granular precipitate. The first account of the precipitate from lysed platelets (13) described it as consisting of both filamentous and amorphous components and showed that it contained a band with the mobility of myosin heavy chain on SDS gels. As we find filaments and the 200-Kdalton band with activated platelets,

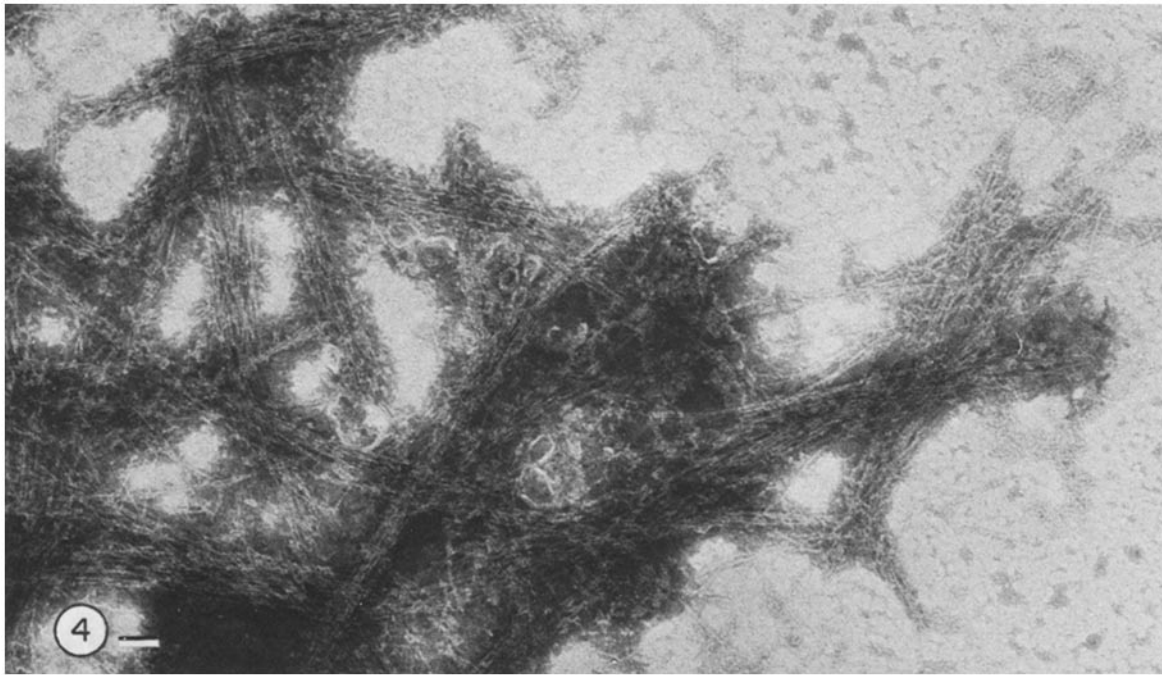
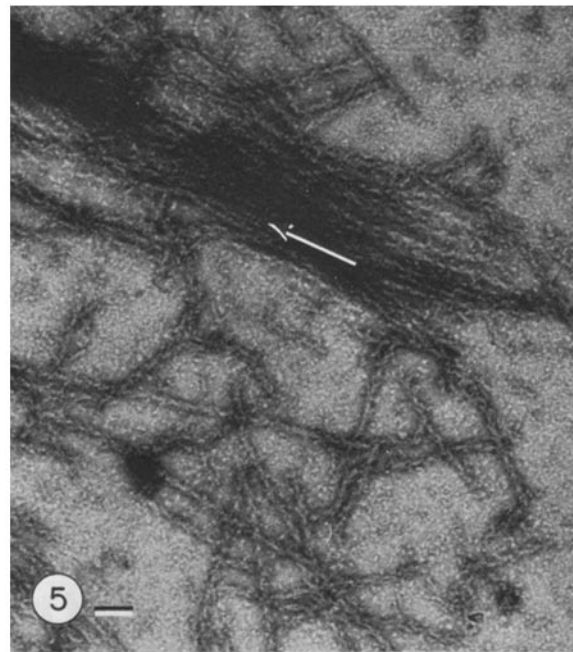


FIGURE 4 Negatively stained bundles of filaments from a transformed precipitate. 2% ammonium molybdate.  $\times 54,000$ .

FIGURE 5 A similar preparation of negatively stained bundles of filaments from a transformed precipitate decorated with subfragment one from muscle myosin. 1% uranyl acetate.  $\times 54,000$ .



and the amorphous precipitate without the 200-Kdalton band from discoid platelets, we believe that these earlier preparations were partially activated. We also obtain a mix of amorphous and filamentous regions and a 200-Kdalton band when platelets are partially activated (Fig. 2). The fact that the precipitates from resting platelets also lacked  $K^+$ ,  $Ca^{++}$  ATPase activity present in the precipitates from activated platelets confirms the absence of myosin. Therefore, myosin seems to have different solubility properties in lysates from resting and activated platelets. This could be caused by changes in actin, or in the myosin itself, in view of a report that myosin is phosphorylated in platelets activated by thrombin (5), and that phosphorylated myosin from lymphocytes has an increased ability to form filaments (20).

It is true that we have only obtained the fully amorphous state by anesthetic treatment. However, we believe that this is not dependent on the treatment in the sense of an artificial

effect but is rather caused by the more complete inhibition of shape change. In individual platelets, the same kind of granular ground cytoplasm is seen after anesthetic treatment or if the platelets are untreated but carefully selected (16, 17). This is not yet possible to do with masses of platelets. Furthermore, the tetracaine treatment at this concentration and time is completely reversible (18).

Although myosin is present in the activated precipitates, our results with in vitro transformation show that it is not necessary for bundle formation. In fact, the only change observed on the gels after bundle formation is induced, is some partial loss of the 100-Kdalton component which has the mobility of  $\alpha$ -actinin, and an almost complete loss of the 35-Kdalton protein. Conversely, the profilin-like component that runs just above hemoglobin (9, 14) on our gels is absent from the precipitate from activated platelets. Yet there does not appear to be a loss of this component during transformation of the precipitate

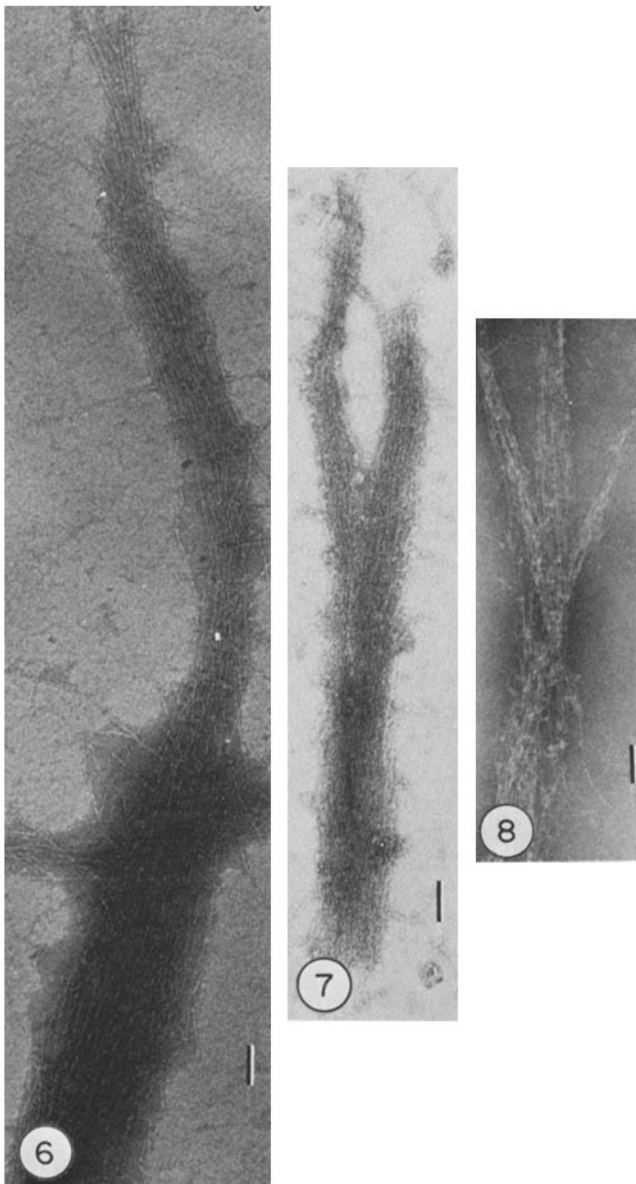


FIGURE 6 A negatively stained bundle from an ionophore-activated platelet.  $\times 54,000$ .

FIGURE 7 A negatively stained bundle from a precipitate transformed in vitro. Compare with Fig. 6.  $\times 54,000$ .

FIGURE 8 Another example of a bundle of microfilaments after in vitro transformation.

from resting platelets. However, problems with nonspecific stickiness, especially with this insoluble and cohesive precipitate, cannot be ruled out. It has also to be considered that the in vitro transformation, which occurs in about one-third to one-half of the precipitate, may not involve a profilin component. Carlsson et al. (3) were not able to convert profilactin alone to filaments with the levels of calcium and the pH that are effective with the precipitate. Further study will be necessary to distinguish these possibilities.

A recent report (23) describes the presence of a 90- to 95-Kdalton protein in human platelets that inhibits actin assembly only in the presence of calcium and appears to be a villin-like protein. This is not the same as the 100-Kdalton protein that is a major component of our precipitate, because a less intense 95-Kdalton band is also present and resolved on our 6% gels as

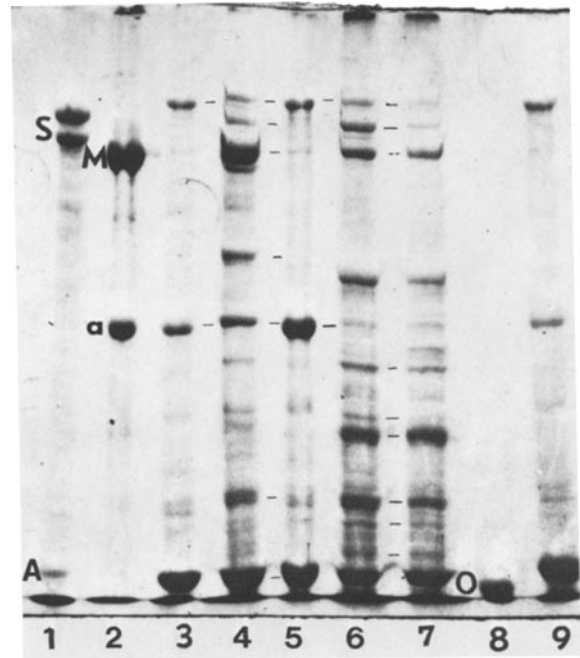


FIGURE 9 SDS gel electrophoresis on 6% acrylamide. Lane 1: spectrin (S) and actin (A) standards; lane 2: myosin (M) and  $\alpha$ -actinin (a) standards; lane 3: precipitate from tetracaine-treated resting platelets; lane 4: precipitate from A-23187-treated platelets. Note the presence of a major new band with the mobility of myosin, retention of the band with the mobility of  $\alpha$ -actinin, and appearance of several minor bands, including one between  $\alpha$ -actinin and myosin, found to be 135 Kdaltons on a staining plot; lane 5: co-electrophoresis of authentic  $\alpha$ -actinin and tetracaine precipitates; lane 6: supernate from tetracaine-activated platelets; lane 7: supernate from A-23187-treated platelets. Note overall similarity of the patterns but decrease in myosin and other bands that are present in the A-23187 precipitate shown in lane 4, suggesting the decrease is not caused by proteolysis; lane 8: ovalbumin standard (O); lane 9: tetracaine precipitate transformed with  $5 \times 10^{-5}$  M  $\text{Ca}^{++}$ , pH 7.6, and washed three times. Note decrease in 100-Kdalton band and compare with lane 3.

shown in Fig. 9.

Preliminary results also show that the 100-Kdalton protein is similar to  $\alpha$ -actinin in two other respects: it cross-reacts with antibody to chicken gizzard  $\alpha$ -actinin and yields a one-dimensional peptide map that is similar to that of  $\alpha$ -actinin (B. Langer, P. A. Gonnella, and V. T. Nachmias, unpublished observations).

Not all the bundles we observe resemble precisely the filament bundles in platelet filopodia: usually they are shorter and thicker, measuring only up to  $\sim 0.8 \mu\text{m}$  long, while filopodia may be several micrometers long. In a few cases we find longer bundles (Fig. 7). However, lengths of filopodia in platelets vary; "spontaneous" ones are often longer than  $1 \mu\text{m}$ ; after activation by ionophore, they are shorter and more numerous. Some of the bundles formed are remarkably similar to platelet filopodia. We appear to have an in vitro analogue of platelet shape change which is now susceptible to further analysis. Schollmeyer and colleagues (21) reported that mixtures of muscle  $\alpha$ -actinin, platelet actin-binding protein, actin, and tropomyosin-troponin resulted in the formation of long bundles of microfilaments. They did not report any divalent ion or pH requirements for such bundles nor discuss stoichiometry, but further comparison of their aggregates with ours will be valuable.



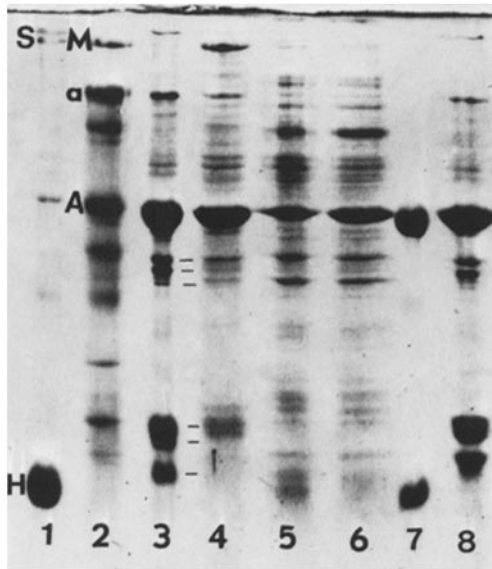


FIGURE 10 SDS gel electrophoresis in 15% acrylamide. Lane 1: spectrin (S), actin (A), and hemoglobin (H) standards; lane 2: myosin (M),  $\alpha$ -actinin (a) and actin standards; lane 3: precipitate from tetracaine-treated resting platelets. Note the presence of a prominent low molecular weight band just above hemoglobin which plots as 16.5 Kdaltons; lane 4: precipitate from A-23187-treated platelets. Note the decrease in the low molecular weight band; lane 5: supernate from resting platelets; lane 6: supernate from activated platelets. Note overall similarity of these two but decrease in 200-Kdalton band in lane 6; lane 7: ovalbumin and hemoglobin standards; lane 8: tetracaine precipitate transformed with  $5 \times 10^{-5}$  M  $Ca^{++}$ , pH 7.6, and washed three times. Note the persistence of the low molecular weight bands and decrease in 100-Kdalton band.

TABLE I  
 $K^+$ ,  $Ca^{++}$  ATPase of Precipitates

exp	Ionophore	Tetracaine
1	0.036 $\mu$ mol/min/ml	0
	0.04 $\mu$ mol/min/ml	0
2	0.06* $\mu$ mol/min/mg	0.002 $\mu$ mol/min/mg

\* If myosin estimated as 10–15% of the proteins on the stained gel, the sp act is 0.4–0.6.

Other instances of transformation of regions of cells containing amorphous precursors into microfilaments have been reported (22, 2, 4). In the acrosomal process of echinoderm sperm and in unfertilized egg cortices, an increase in pH was adequate to cause microfilament polymerization. Preliminary results suggest that pH increase can lead to free filaments in the precipitates, but in our preparation pH alone did not cause bundles to form.

It has been pointed out that the evidence for an increase in free calcium ion during platelet activation is circumstantial (6). Our findings suggest that intracellular pH change is of primary importance in the earliest stage of activation, i.e., shape change. Platelets are so small that relatively few ions could cause large shifts in hydrogen ion concentration. Recently, Leven and Nachmias (12) reported that megakaryocytes, whose volume is about one thousand times that of their progeny, undergo a specific spreading reaction when treated with adenosine di-

phosphate, a potent activator of platelet shape change. It is relevant to the present discussion that this spreading reaction can be mimicked by A-23187 only if methylamine is also used (12), and methylamine alone is effective in a certain percentage of cases. Methylamine enters the cytoplasm of cells presumably as the unprotonated form (7) which can then lead to increased internal pH if present in high concentration.

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