The interaction of platelet actin, myosin and myosin light chain kinase

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Abstract  Human blood platelets contain two proteins very similar in structure and function to the contractile proteins of muscle: actin and myosin. Platelet actin has a similar molecular weight (43000 daltons) and amino acid composition to muscle actin. It polymerizes into long filaments which can form 'arrowheads' with skeletal muscle and platelet myosin subfragment-1 (S-1) as viewed in the electron microscope. These 'arrowheads' are dissociated by Mg-ATP. Platelet actin activates the ATPase activity of muscle heavy meromyosin to approximately the same extent as muscle actin.

Platelet myosin (molecular weight 460 000), like muscle myosin, is composed of heavy chains (200 000 daltons) and light chains (20 000 and 16 000 daltons). The light chains resemble those found in other cytoplasmic (non-muscle) myosins and smooth muscle myosin in charge (at pH 8.4) and size and differ from the light chains of skeletal muscle and cardiac myosin.

Human platelets contain a kinase that transfers the terminal phosphate from γ-labelled ATP32P to the 20 000 dalton light chain of platelet myosin. When platelet myosin is phosphorylated, its actin-activated ATPase activity is markedly increased. Moreover, if phosphorylated myosin is dephosphorylated with E. coli alkaline phosphatase, its actin-activated ATPase activity is decreased. These findings indicate that the phosphorylation–dephosphorylation of platelet myosin is a major controlling factor in platelet actin–myosin interaction. The ability of platelet myosin kinase to phosphorylate myosin from fibroblast and smooth muscle cells suggests that myosin phosphorylation may play a functional role in other cells.

Human blood platelets contain proteins similar in structure and function to the muscle proteins actin and myosin (Bettex-Galland & Lüischer 1965; Booyse et al. 1971; Adelstein & Conti 1972; Booyse et al. 1973). Indeed, it is now known that cytoplasmic actin and myosin are present in a large variety of non-muscle cells from human and other vertebrate and non-vertebrate systems (for a review on the subject of cytoplasmic actin and myosin, see Pollard & Weihing 1974).

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Our laboratory has been studying platelet myosin, actin and a recently isolated enzyme, platelet myosin light chain kinase, in order to understand the interaction of these proteins in platelets. The purpose of these studies is to discover the role these proteins play in specialized cell functions such as the platelet 'release reaction' and clot retraction as well as their possible role in functions common to all cells, such as motility and cell division.

In this paper we briefly review the structural and chemical properties of platelet actin and myosin. We shall then discuss the following properties of these proteins: (1) the phosphorylation of platelet myosin by a recently purified endogenous kinase, (2) the use of this platelet kinase to phosphorylate other cytoplasmic and smooth muscle myosins, (3) the effect of phosphorylation on the interaction of platelet actin and myosin.

PLATELET ACTIN

A number of methods for purifying platelet actin have been described (Adelstein & Conti 1972; Booyse et al. 1973). Human platelet actin has a molecular weight (43 000 daltons) and an amino acid composition similar to muscle actin. This includes the presence of 1 mole per molecule of the unusual amino acid N\(^\text{\textdegree}\)-methyl histidine. Recent studies in the laboratory of Dr Marshall Elzinga (unpublished) on the cyanogen bromide fragments of platelet actin indicate identical amino acid sequences for some of the platelet actin and rabbit skeletal muscle actin peptides (Elzinga et al. 1973). There are a few discrete amino acid replacements which might reflect either a species difference or a difference between cytoplasmic and muscle actin.

Recently, Lazarides & Weber (1974) showed that non-muscle and muscle actin have similar antigenic determinants. They prepared antibodies to mouse fibroblast actin eluted from sodium dodecyl sulphate–polyacrylamide gels and found that these antibodies cross-reacted with chicken muscle actin.

Studies on the function of platelet actin are less complete. We have shown that human platelet actin activates rabbit skeletal muscle heavy meromyosin (HMM) to approximately the same extent as rabbit muscle actin. The activation measured at low ionic strength and 37 °C was from 0.1 to 1.5 μmoles P\(_{i}\)/mg protein per min. Skeletal muscle troponin–tropomyosin conferred Ca\(^{2+}\) sensitivity on this system (Adelstein & Conti 1972). Lower activation levels reported by others for cytoplasmic actins (Pollard & Weihing 1974) may reflect partial denaturation of the protein.

The process of polymerization, transforming platelet G-actin (globular) to F-actin (fibrous) in the presence of 0.1 M-KCl and 2 mM-MgCl\(_2\), is known to
occur but has not been as well studied as in the case of muscle actin (Oosawa & Kasai 1971). We have noted a marked pH dependence for this process in the platelet protein, the optimum being pH 7.5. Polymerized platelet actin is capable of forming 'arrowheads' with platelet myosin subfragment-1 (S-1), as well as skeletal muscle myosin S-1, as viewed in the electron microscope. These 'arrowheads' can be dissociated by Mg-ATP (Adelstein et al. 1971).

In a recent study on actin isolated from neutrophilic polymorphonuclear leucocytes, Boxer et al. (1974) reported that actin from leucocytes with subnormal locomotion and ingestion capabilities failed to polymerize under conditions that fully polymerized normal leucocyte actin.

The existence of a second form of platelet actin has been described by Probst & Lüscher (1972) and Abramowitz et al. (1975). This second form of actin, similar in amino acid content to the primary form described above, remains depolymerized under conditions leading to the polymerization of ordinary platelet actin. It has been postulated that this actin may play a role in determining cell shape, that unlike the usual form of actin it does not interact with platelet myosin, but that it may undergo polymerization–depolymerization inside the platelet.

PLATELET MYOSIN

Platelet myosin has a molecular weight of approximately 460 000 and is composed of two heavy chains (200 000 daltons) and two different light chains (20 000 and 16 000 daltons). We have isolated the intact molecule as well as the proteolytic fragments S-1 and rod (see Fig. 1). As is the case for skeletal muscle myosin, the platelet myosin S-1 fragment retains the ATPase activity as well as the ability to bind reversibly to actin. The rod portion retains the ability to form bare thick filaments at low ionic strength (Adelstein et al. 1971).
The two light chains of platelet myosin are similar in molecular weight to the light chains of other cytoplasmic (e.g., fibroblast) and smooth muscle myosins. They also resemble these light chains in charge, as evidenced by their electrophoretic mobility, in polyacrylamide-urea gels (pH 8.4). On the other hand, the platelet light chains differ in these parameters from the light chains of skeletal and cardiac muscle myosin (Adelstein & Conti 1974).

Although platelet myosin and smooth muscle myosin have similar light chains, Willingham et al. (1974) have immunological evidence for differences in the heavy chains. Antibodies to fibroblast myosin cross-react with the isolated rod portion of the platelet myosin molecule (for the isolation of platelet rod, see Adelstein et al. 1971). These particular antibodies fail to cross-react with smooth muscle myosin, indicating a difference in the rod portion of platelet and smooth muscle myosin.

In summary, platelet myosin resembles other cytoplasmic myosins as well as smooth muscle myosin in containing a similar (possibly identical) complement of light chains. These light chains differ from those of skeletal and cardiac muscle myosin. At least part of the rod portion of the heavy chains of platelet and fibroblast myosin differs from that of smooth muscle myosin.

Recent experiments with antibodies to non-muscle myosin indicate a potential method for localizing myosin in platelet cells. Using antibodies to fibroblast myosin, Willingham et al. (1974) showed that a part of the fibroblast myosin is localized on the outside of the fibroblast cell membrane. Weber & Groeschel-Stewart (1974) prepared antibodies to chicken gizzard myosin which they used to locate cytoplasmic myosin inside fibroblast cells from human, mouse and chicken sources. It is of interest that when Willingham et al. (1974) made antibodies to smooth muscle myosin (mouse uterus) they failed to cross-react with mouse fibroblast myosin, although they did cross-react with chicken gizzard myosin. Before firm conclusions can be drawn about myosin localization it must definitely be established: (a) that the myosin being utilized in antibody production is pure by a number of criteria and (b) that the antibodies produced not only cross-react with a cellular component in situ, but also cross-react with myosin isolated from the cell.

Table 1 summarizes the enzymic activity of platelet myosin at high ionic strength and compares it to that of smooth, skeletal and cardiac myosin. As is the case with the light chains, the ATPase activity of platelet myosin resembles that of smooth muscle more than cardiac and skeletal muscle myosin. This is also true for the actin-activated ATPase activity measured in the presence of Mg$^{2+}$ at low ionic strength. This last parameter is thought to be the most physiologically relevant of the in vitro enzymic measurements, and we shall return to it below in discussing the effect of platelet myosin phosphorylation.
TABLE 1

ATPase specific activity of platelet and muscle myosin

<table>
<thead>
<tr>
<th></th>
<th>ATPase specific activity (μmol Pi/mg protein per min)</th>
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<tr>
<td></td>
<td>K⁺-EDTA</td>
</tr>
<tr>
<td>Non-muscle</td>
<td></td>
</tr>
<tr>
<td>Human platelet</td>
<td>1.4</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
</tr>
<tr>
<td>Horse smooth</td>
<td>1.37</td>
</tr>
<tr>
<td>Rabbit skeletal (white)</td>
<td>4.2</td>
</tr>
<tr>
<td>Human cardiac</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Assay conditions: 20 mM-Tris-HCl (pH 7.2), 2 mM-EDTA or 10 mM-CaCl₂; 0.5 M-KCl, 2 mM-ATP, 0.25 mg/ml myosin, 37 °C.

In an effort to understand the factors controlling actin–myosin interaction in the platelet as well as other non-muscle cells, we began a study on platelet protein phosphorylation. Since kinases are often bound to their substrate, our initial studies involved the addition of γ-labelled AT³²P to a high salt (0.5 M-KCl) extract of lysed platelets at pH 7.5 (see Fig. 2).

The only protein phosphorylated in this extract is myosin, specifically the 20000 dalton light chain (Adelstein et al. 1973). This corresponds to the findings of Perrie et al. (1973) who phosphorylated the 18500 dalton light chain.
chain of rabbit skeletal muscle myosin using an exogenous kinase. As mentioned above, this light chain differs both in size and charge from the phosphorylated platelet light chain.

The original experiments on myosin phosphorylation in a platelet extract revealed that $^{32}P$ could be utilized from $\gamma$-labelled AT$^{32}P$ (and not GT$^{32}P$ or $^{32}P_i$) to form a stable covalent (ester type) bond (see Fig. 2). Subsequently, we found that if fresh intact platelets are incubated in the presence of $^{32}P_i$, small but significant quantities of $^{32}P_i$ are incorporated into the 20 000 dalton light chain of the purified myosin. This suggests that $^{32}P$ is first incorporated into the platelet pool of ATP and is then used to phosphorylate the myosin light chain. We are now repeating this $^{32}P_i$ incubation in the presence of ADP, thrombin and dibutyryl cyclic AMP to see if these reagents, known to affect platelet aggregation, have any effect on the incorporation of $^{32}P_i$ into platelet myosin.

**PLATELET MYOSIN LIGHT CHAIN KINASE**

The enzyme was purified from human platelets by an initial extraction at low ionic strength to separate it from myosin, and subsequent column chromatography on DEAE-Sephadex, Biogel P-200 and hydroxyapatite (for details of purification, see Daniel et al. 1974 and Adelstein et al. 1975). This procedure resulted in an approximately 500-fold purification of the kinase. The enzyme was not purified to homogeneity since approximately seven bands were seen on polyacrylamide gel electrophoresis (done in the absence of SDS in order to preserve the native enzyme structure), only one of which possessed enzymic activity after elution from the gel.

The properties of the purified enzyme are summarized in Table 2. It is similar to the skeletal muscle kinase purified by Pires et al. (1974) in size and apparent independence of cyclic AMP for its phosphorylating activity. Unlike the skeletal muscle enzyme, the platelet kinase has no Ca$^{2+}$ requirement but does require Mg$^{2+}$ for its activity.

**SUBSTRATES**

The substrate specificity of the platelet kinase is quite interesting, particularly since it follows the structural and functional similarities of the myosins outlined above (Table 2). The platelet kinase phosphorylates the 20 000 dalton light chain of mouse fibroblast and chicken gizzard myosin. The platelet kinase is inhibited by rabbit and human skeletal muscle myosin and cannot phosphorylate human cardiac myosin. The inhibition by skeletal muscle myosin suggests a
Table 2: Properties of platelet myosin kinase

<table>
<thead>
<tr>
<th>Property</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>83,000 ± 10%</td>
</tr>
<tr>
<td>Cation dependence</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td>Substrates:</td>
<td></td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>human platelet, mouse fibroblast, chicken gizzard, mouse uterus</td>
</tr>
<tr>
<td></td>
<td>20,000 dalton light chain</td>
</tr>
<tr>
<td>No phosphorylation</td>
<td>rabbit skeletal muscle, human skeletal muscle, human cardiac muscle, canine cardiac muscle</td>
</tr>
</tbody>
</table>

Basic similarity in the site to which the enzyme can bind without being able to transfer the phosphate group. The finding that the platelet kinase can phosphorylate another cytoplasmic myosin (i.e., fibroblast) suggests that the process of phosphorylation may extend to myosins in cells other than platelets. Indeed, we have used the platelet enzyme as a probe in an effort to uncover the presence of non-muscle myosin in human rhabdomyosarcoma cells.

At present, we are investigating the hypothesis that tumour cells contain significant amounts of cytoplasmic myosin which functions in cell division. We chose the malignant skeletal muscle tumour rhabdomyosarcoma to see if these cells contain normal skeletal muscle myosin as well as cytoplasmic myosin. Our working hypothesis is that cytoplasmic myosin, which is involved in cell division, would be present in unusually large amounts in neoplastic rhabdomyosarcoma cells. Since some of these cells continue to show evidence of myofibrils, we would also expect to isolate skeletal muscle myosin. We hoped to differentiate muscle and cytoplasmic myosin by the ability of the latter to be phosphorylated by platelet kinase as well as by the size and charge of the light chains.

Myosin prepared from the excised tumour of a 4-year-old girl was found to contain a mixture of human skeletal and cytoplasmic myosin (see Fig. 3). Because cytoplasmic myosin only partially dissociates from actin under conditions which completely dissociate skeletal muscle actomyosin (i.e., the presence of Mg-ATP), the two myosins could be separated by chromatography on Sepharose 4B. Incubation of cytoplasmic myosin with platelet myosin kinase and [$\gamma$-$^{32}$P]ATP resulted in phosphorylation of the 20,000 dalton light chain, whereas the enzyme had no effect on the normal human skeletal muscle myosin.
FIG. 3. Schematic representation of 7.5% polyacrylamide-sodium dodecyl sulphate gels of rhabdomyosarcoma and human skeletal myosin. (a) rhabdomyosarcoma myosin before Sepharose chromatography, (b) cytoplasmic myosin purified from a by Sepharose chromatography, (c) human skeletal myosin with trace cytoplasmic myosin, purified from a, (d) control sample of human skeletal myosin. HC, heavy chain; A, actin; LC, light chains. *Phosphorylated light chain.

(see Fig. 3). Further studies will be necessary to ascertain that the tumour cells are the source of both myosins. Dr Donald Henson and Rachel Levinson (National Cancer Institute) are using antibodies to both human skeletal and fibroblast myosin to study the localization of myosin in sections made from the same tumour. Of relevance is our finding that a pure line of human rhabdomyosarcoma cells (A204), grown in vitro and showing no evidence of myofibrillar formation, contain light chains of cytoplasmic myosin (i.e., 20 000 and 16 000 dalton) and not human skeletal muscle myosin (25 000 and 18 500).

THE ROLE OF PHOSPHORYLATION

The effect of phosphorylating platelet myosin is to increase the actin-activated ATPase activity measured in the presence of Mg\(^{2+}\) at low ionic strength (see Table 3) (Adelstein et al. 1975). Phosphorylation has no effect on the myosin ATPase activity measured in the presence of K\(^{+}\)-EDTA at high ionic strength. Table 3 shows that myosin, before phosphorylation, can be activated to some extent but the resulting specific activity is one-fifth the specific activity of actin-activated phosphorylated myosin. We do not yet know if this low level of activation is an inherent property of the myosin or reflects partial phosphorylation of the myosin during the purification procedure.

Although a platelet phosphatase which dephosphorylates platelet myosin has yet to be isolated, we have succeeded in partially dephosphorylating
TABLE 3

The effect of phosphorylation on ATPase specific activity

<table>
<thead>
<tr>
<th></th>
<th>ATPase specific activity (μmol P_i/mg protein per min)</th>
<th>Actin-activateda</th>
<th>+ Actin</th>
<th>K⁺-EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control myosin</td>
<td>0.002</td>
<td>0.020</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>Phosphorylated myosin</td>
<td>0.009</td>
<td>0.100</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

a Assay conditions: 10 mM-Tris-HCl (pH 7.2), 1.4 mM-MgCl₂, 1 mM-ATP, 30 mM-KCl, 0.13 mg/ml myosin, 1.2 mg/ml actin, 37 °C.

b Assay conditions: 20 mM-Tris-HCl (pH 7.2), 2 mM-EDTA, 0.5 mM-KCl; 2 mM-ATP, 0.25 mg/ml myosin 37 °C. (For details of assay, see Adelstein et al. 1971. For details of phosphorylation, see Adelstein et al. 1975.)

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Fig. 4. Release of ³²P from phosphorylated myosin incubated with alkaline phosphatase. ³²P-labelled phosphorylated myosin was incubated with alkaline phosphatase and samples removed at the times indicated for (●) determination of ³²P remaining and (×) actin-activated ATPase specific activity remaining. The upper line (control) shows that counts were not lost from a control sample incubated under the same condition without alkaline phosphatase. The phosphorylated myosin contained 30,000 c.p.m./0.05 mg of protein and an actin-activated ATPase specific activity of 0.12 μmol P_i/mg myosin per min.

platelet myosin using *E. coli* alkaline phosphatase (Adelstein et al. 1974; Conti & Adelstein 1975). Fig. 4 illustrates a time course for the release of ³²P from previously phosphorylated platelet myosin in the presence of alkaline phos-
The actin-activated ATPase activity for the appropriate time is plotted on the same graph. Dephosphorylation is accompanied by a decrease in the actin-activated myosin ATPase activity but, like phosphorylation, it has no effect on the K⁺-EDTA and Ca²⁺-activated myosin ATPase activity measured at high ionic strength.

These studies suggest that phosphorylation acts as a switch that turns on myosin so that it can interact, in a physiologically meaningful way, with actin. Since interaction with actin (detected by an increase in the actin-activated ATPase activity) does not result in dephosphorylation, the switch evidently remains on until a phosphatase turns it off by dephosphorylating myosin. The ability of the platelet enzyme to phosphorylate other non-muscle myosins, such as fibroblast myosin and chicken gizzard myosin, suggests that this process may play a role in the numerous other non-muscle cells in which myosin and actin have been detected, as well as in smooth muscle.

Phosphorylation does not appear to confer Ca²⁺ sensitivity on the platelet actin–myosin system. This means that phosphorylated myosin is activated by actin both in the presence and absence of Ca²⁺. In muscle, the troponin–tropomyosin complex confers Ca²⁺ sensitivity. It is possible that this fine tuning of the switch is also conferred by troponin–tropomyosin in the platelets (Cohen et al. 1973). The identification of platelet tropomyosin has been described by Cohen & Cohen (1972). Positive identification of platelet and other cytoplasmic troponins has yet to be accomplished.

Both general and specialized cell functions may be affected by the interaction of actin with phosphorylated myosin. Specialized cell functions in the platelet include the process of clot retraction and platelet aggregation. In secretory cells, specialized functions involve the release of substances such as hormones and neurotransmitters (Puszkin & Kochwa 1974). General cellular processes that might be affected by this interaction include cell division, as discussed above in the case of rhabdomyosarcoma cells, and cell migration, which is of particular importance in blood cells, as well as embryonic cells, particularly during the earliest stages of development. The challenge of these speculations awaits the proof of further experimentation.

SUMMARY

(1) Human blood platelets contain myosin which is similar in structure and function to smooth muscle myosin and to cytoplasmic myosins from other non-muscle sources.

(2) Platelet myosin light chain kinase can phosphorylate the 20 000 dalton light chain of platelet myosin, fibroblast myosin and chicken gizzard myosin.
(3) The effect of phosphorylation is to increase the actin-activated ATPase activity of platelet myosin.

(4) Phosphorylation might play a similar role in controlling actin–myosin interaction in other cells found to contain these proteins.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the help of Dr Donald Henson and Rachel Levinson for growing the A204 rhabdomyosarcoma cells and Dr John Ziegler for the rhabdomyosarcoma surgical specimens. We also thank Mrs Kathy Knudsen for typing the manuscript.

References


Discussion

Nachman: You discussed the antigenic relationships between the various actomyosins. Becker and I, using actomyosin from a human pregnant uterus, made an antibody in rabbits (Becker & Nachman 1973). This antibody to smooth muscle actomyosin stains human megakaryocytes and platelets. An antibody to crude thrombosthenin, which contains tropomyosin, actin and all the myosin fragments, stains human uterus. By immunodiffusion there is partial identity between thrombosthenin and uterine actomyosin. So in the human system there is clear-cut evidence of an antigenic relationship between platelet actomyosin and uterine actomyosin.

Adelstein: Two laboratories working with non-muscle myosin have found that antibodies to these myosins fail to cross-react with smooth muscle myosin. Willingham et al. (1974) found that antibodies to mouse fibroblast myosin failed to cross-react with mouse uterus myosin, and Pollard found that antibodies to human platelet myosin did not cross-react with human uterus myosin (T. D. Pollard, personal communication).

On the other hand, antibodies to mouse fibroblast myosin do cross-react with human platelet myosin, specifically with the rod portion of the molecule (M. C. Willingham & R. S. Adelstein, unpublished work).
These findings do not exclude the possibility that cytoplasmic (non-muscle) myosin may have some antigenic sites in common with smooth muscle myosin which would result in a population of antibodies that would cross-react with both myosins. I would simply like to point out that one must always be careful about the purity of the antigen—in this case, the myosin.

Cohen: Dr Adelstein described some of the similarities and differences among myosins from platelets and muscle cells. We have also studied the properties of platelet myosin, particularly of platelet light meromyosin (LMM), obtained by splitting the myosin molecule with trypsin. We find an axial periodicity of 14.5 nm (145Å) in tactoids of platelet LMM, which is similar to the periodicity of LMM from smooth and skeletal muscle. This may indicate a fundamental similarity in the structure of these molecules, at least in respect of their assembly.

The most striking difference between muscle and platelet systems lies in the tropomysin molecule. Platelet tropomysin is smaller than other, muscle tropomysins. This two-chain α-helical molecule has a subunit molecular weight of 30,000 and forms paracrystals with an axial period of 34.5 nm, which is close to the length of the molecule (Cohen & Cohen 1972). It is therefore smaller in size than the 40 nm long tropomysins from muscle. Tropomysin may be involved in the regulation of contraction which we have demonstrated to be of the actin-linked type in platelets (Cohen et al. 1973). Fine et al. (1973) have shown characteristics for tropomysin from brain tissue similar to those of platelet tropomysin. This may imply distinctive structural and spatial relationships in the contractile elements of non-muscular systems, as compared with muscle systems.

Dr Adelstein, you discussed the function of phosphorylation in the platelet system and mentioned that the actin activation of myosin is increased by phosphorylation. Compared to that of skeletal muscle myosin, the actin activation of platelet myosin is rather weak. Pollard (1975) has shown that the actin activation of platelet myosin is increased by the presence of a co-factor, which he has not yet completely identified. Could this co-factor be related to a protein kinase?

Adelstein: At present, since the co-factor has not been purified, it is not possible to determine whether it might be a kinase. However, the following points should be made: (a) Pollard's co-factor was isolated from rabbit skeletal muscle and added to platelet myosin; (b) the actin-activated ATPase activity found in the presence of the co-factor is the same order of magnitude as that found by us for phosphorylated myosin (T. D. Pollard, personal communication). Until the co-factor is at least partially purified it is not possible to know whether it is a kinase or not.
**Haslam:** Perhaps it is not surprising that cyclic AMP did not activate the phosphorylation of the myosin light chains, Dr Adelstein. As you found an increase in actin-activated ATPase after phosphorylation and some evidence that ADP increases the phosphorylation of platelet myosin, it is likely that, if anything, increased phosphorylation would be associated with decreased cyclic AMP levels. This raises the question of whether myosin light chain kinase is activated by cyclic GMP, which does increase in response to ADP (see my paper, Fig. 7, p. 135).

**Adelstein:** We have tried cyclic GMP and not found any effect, but the experiment needs to be repeated. Our original thought was that cyclic AMP actually decreased the kinase activity, but we have not yet substantiated this. Our experiment using $^{32}$P and ADP, which causes aggregation, has only been done once and must be repeated.

**Crawford:** Do you always, in your myosin activation experiments, titrate to maximal response? In other words, do you establish the most favourable combining ratio for the actin and myosin?

**Adelstein:** Yes; we have had to do that.

**Crawford:** It occurs to me that some of the activation of the myosin by actin may depend upon a number of factors which are difficult to control experimentally—for example, variability in the length and polarity of the actin polymer and whether or not it is present as the two-stranded helical structure you get in muscle or as a single-stranded Mg$^{2+}$ polymer, of the kind seen in *Physarum* and *Dictyostelium* (Hatano & Oosawa 1966; Woolley 1972). There may even be lateral association of the polymeric units. All these factors could influence the combining properties of the two polymers and I would think it would be extremely difficult to define such interactions in quantitative terms.

**Adelstein:** We are using rabbit skeletal muscle actin which has been polymerized in the presence of 30 mM-KCl, 2 mM-MgCl$_2$, 7 mM-Tris- HCl (pH 7.5) and 2.5 mM-DTT. We use a 10-15-fold excess of actin (mg/mg) over platelet myosin. Platelet actin polymerized under these conditions appears to activate platelet myosin to the same extent (R. S. Adelstein & M. A. Conti, unpublished results).

**Detwiler:** We have been interested in these systems for some time, and one problem relates to the preparation of the platelet actin. I wonder what your normal yield of polymerizable actin is and whether you are dealing with a very small fraction of the total actin of the cell, or a substantial part of it. I am not specifically referring to the second form of actin that we described (Abramowitz et al. 1975), but if you go through the conventional procedures for preparing actin, you obtain only a small amount of material. I am worried by discussions of actin-activated myosin ATPase when platelet actin is used; it might be just...
some small part of the total actin that is capable of this activity.

Adelstein: In the experiments on the actin-activated ATPase activity we
purify platelet myosin free of platelet actin and in most cases we add back
rabbit skeletal muscle actin. We have also added back platelet actin but the
procedure we employ for isolating platelet actin makes use of the ability of
actin to polymerize at high ionic strength (Adelstein & Conti 1972).

We have never quantified how much platelet actin can be polymerized but
we do know that unlike rabbit skeletal muscle actin, the polymerization of
platelet actin is very sensitive to pH. Platelet actin, shown to be polymerized
at pH 7.5 by both flow birefringence and increased viscosity, was depolymerized
by simply raising the pH to 8.3. Polymerization was restored by lowering the
pH to 7.5 (Adelstein & Conti 1972).

Lüscher: The yields of purified actin isolated from crude platelet actomyosin
are certainly deplorably low. However, once actin is in the fibrillar F form, it
can be depolymerized and repolymerized with a 95% yield.

Crawford: There are also difficulties with conventional acetone powder
preparations of whole platelets. The extractability of the actin from the powder
is quite low and you certainly leave some actin behind, probably a considerable
proportion. I suspect, since in our own cell fractionation studies that I referred
to earlier (p. 42) the cytosol actin seems not to combine with skeletal muscle
myosin, that the low ionic strength extraction of acetone powder probably
favours the extraction of this non-combinable actin. This may be G-actin or
perhaps an effete monomer which is no longer polymerizable under any con-
ditions. Perhaps, in fact, we leave the contractile-competent platelet actin in
the acetone powder debris, protected from extraction by its association with
the particulate elements of the cell.

Lüscher: It remains most intriguing that there is no electron microscopical
evidence for the presence of pre-formed cytoplasmic actin microfibrils in a
resting platelet. These become immediately discernible upon stimulation. In
what form is the actin present in resting platelets?

Adelstein: Without knowing for sure, I would favour the existence of small
polymers which would polymerize into long fibrous polymers under the right
stimulus.

Lüscher: Thus one is faced with the interesting situation that in the platelet,
unlike for example striated muscle, there is no evidence for a morphologically
defined contractile structure which can as such be stimulated into activity. In
order to have contractile activity, it is necessary to assemble such a system
first.

Adelstein: A current problem in studies of smooth muscle, similar to one
that exists in non-muscle contractile systems, is the positive identification of
troponin. Troponin is a complex of proteins that together with tropomyosin confers calcium sensitivity on skeletal muscle actomyosin; that is to say, actin-activation of the myosin ATPase activity will occur only in the presence of Ca^{2+}. In a recent article Bremel (1974) suggests that troponin may not be present in smooth muscle and that calcium sensitivity may be conferred by myosin, similar to the findings of Lehman et al. (1972) in molluscs.

Cohen: I agree with you about the likelihood of the existence of small oligomers of actin in the intact cell. These would grow into long filaments when the cell is stimulated by some as yet unknown mechanism. You might have tropomyosin bound to these small actin oligomers, as only seven actin molecules are needed to provide a site of attachment for one tropomyosin molecule. Seven actin molecules would make quite a small oligomer.

Detwiler: May I pose a general question related to platelet contractile proteins? Dr Lüscher initially pointed out the very large amount of actomyosin in platelets and in the literature one frequently encounters statements about the function of this protein (cf. Bettex-Galland & Lüscher 1965). But to my mind there is no real evidence for any function of platelet contractile proteins. Can anyone tell me what the possible functions of these proteins are, and defend these suggestions with some evidence?

Lüscher: Of course one could argue that the relatively large amount of contractile protein in platelets is simply due to the fact that the megakaryocyte is an extremely mobile cell and that therefore the platelet cannot help containing a lot of actomyosin! There is also, of course, another answer to your question. Microscopical observation of spontaneous haemostasis shows that first a relatively fragile platelet mass is formed, which is barely able to resist the eroding forces of the streaming blood. This loose aggregate suddenly becomes solid and impermeable. This striking phenomenon certainly is not due to the formation of fibrin but rather to active contraction of the aggregate. In fact, in vitro, platelet aggregates will contract at 37 °C within two to three minutes, which corresponds roughly to a normal bleeding time. I think this consolidation of a haemostatic plug, or of a platelet thrombus, is a most important manifestation of contractility.

Furthermore, long spikes form early during the shape change, and later can be retracted completely. Extrusion as well as the withdrawal of these pseudopods certainly are again manifestations of contractility.

Detwiler: You are saying that these are phenomena that are easily attributed to a contractile protein. Intuitively it seems that that would be a nice function of the contractile protein, but there is really no evidence that it is the contractile protein doing that.
Liischer: I really have difficulties in offering any other explanation for the active contraction of a platelet aggregate or for clot retraction.

Born: One way to analyse the retraction of the spikes is to isolate them; we have been trying to do this but it is very difficult. Dr Liischer is right. These cells can pack together almost as tightly as is theoretically possible for them (Born & Hume 1967); the intercellular space becomes very small indeed, and there must be some mechanism for deforming these cells so that they can pack in this way. But what evidence is there about this mechanism?

Feinberg: How would Dr Detwiler explain all the observed movements—spike formation and so on—without contractile proteins?

Detwiler: I couldn’t, but a biophysicist could talk about the extrusion of water from a gel and things of that sort which do not necessarily involve contractile protein. The point is that lack of an obvious alternative explanation is not proof of anything.

Cohen: I don’t think we have syneresis here. We do have a contractile phenomenon, as Budtz-Olsen (1951) has shown by comparing the kinetics of syneresis and clot retraction. So it is not just water coming out.

Haslam: What we would like to have is a specific inhibitor of the action of thrombosthenin, so that we could then identify unequivocally all the processes in which thrombosthenin is involved. I don’t know of any such compound. Thiol reagents, which are usually non-specific, do react with thrombosthenin and do block these processes. What sort of progress has been made towards synthesizing a useful specific irreversible inhibitor of myosin ATPase? Yount et al. (1972) described irreversible inhibition by a purine disulphide analogue of ATP, but this sort of compound would not be able to penetrate the platelet membrane because of its negative charge.

Cohen: I would suggest the use of cytochalasin B. This fungal drug inhibits contraction by disorganizing microfilaments. It also inhibits shape change, and I believe that shape change is a manifestation of contraction.

Haslam: There are problems here. The evidence that cytochalasin B is a sufficiently specific inhibitor of contractile processes is unconvincing. Cytochalasin B is also an extremely potent inhibitor of the transport of sugars and nucleosides across cell membranes and may therefore interfere with cell metabolism. Even the concentrations of cytochalasin B which potentiate the release reaction (i.e. less than 2 μM with washed platelets) can inhibit membrane transport. One could explain this by postulating that there is some connection between the insertion of microfilaments into the membrane and the transport mechanism, so that both are disrupted at the same time. However, at the moment we really do not know enough about how cytochalasin B acts to be able to draw any firm conclusions.
Cohen: J. G. White (1971) has shown that cytochalasin B inhibits shape change, but primary aggregation is unaffected and occurs between disk-shaped platelets.

Haslam: In our hands (Haslam et al. 1975) this inhibitory effect of cytochalasin B on platelet shape change was seen only with high concentrations (e.g. 40 μM in platelet-rich plasma). When you have an inhibitory action of cytochalasin B the possibility of its being metabolic in origin requires investigation.

Cohen: You can reverse the effect of cytochalasin B; you can wash it out and the platelets regain their normal function.

Haslam: The metabolic effects of an inhibition of membrane transport processes may also be reversible.

Lüscher: I would fully subscribe to your doubts, Dr Haslam, because, for instance, cytochalasin B is unable to inhibit the so-called 'superprecipitation' of actomyosin, generally considered to mimic contraction under *in vitro* conditions.

Mustard: I am a bit confused; Dr Detwiler asked a question, to which he received a partial answer, which suggested that when platelets change their shape and form spikes the contractile protein is involved in the shape change. I find that somewhat inconsistent. If the contractile protein restores the platelet disk shape, is it also causing the shape change?

Born: We are using 'contractile protein' in too loose a sense here. There is evidence now for both the sliding mechanism of striated muscle and proteins which contract through a change in conformation.

Lüscher: One has to keep in mind that besides contractile protein which is dispersed in the cytoplasm, the platelet contains preformed contractile material, sometimes termed submembranous fibrils (Zucker-Franklin 1970), just beneath the cell membrane. Since it is likely that calcium first becomes available in the vicinity of the membrane, be it by influx or from storage organelles localized along the microtubules, it is conceivable that these submembranous fibrils will be the first to contract. The formation of the long spikes is then perhaps explained as resulting from the compression of the platelet's cell body. Next, the cytoplasmic contractile system will become organized and activated.

It is only then that contraction of the pseudopods will start. Thus, it is the time-sequence of activation of different compartments of contractile material which determines the course of the morphological manifestations of contractility.

Mustard: To make that explanation acceptable you would have to postulate that the membrane system is not able to contract uniformly; it would have to contract in a differential manner, with parts that contract and parts that do not contract.
Cohen: This is how locomotion happens in amoebae, where cyclic changes occur with parts of the cell in a contracted state and parts in a relaxed state (Taylor et al. 1973).

References


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