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CHARACTERIZATION AND ATPase ACTIVITY OF HUMAN PLATELET ACTOMYOSIN *

J. Lindemans, B.N. Bouma and J.J. Sixma

Division of haemostasis, Department of Internal Medicine,
University Hospital, Utrecht, The Netherlands.

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ABSTRACT

Platelet actomyosin, partially purified by successive precipitation had a specific viscosity of 0,15 and a sensitivity to ATP of 60 %. The enzyme preparation was separated into the actin and myosin components and some myosin fragments by SDS-polyacrylamide gel electrophoresis. The ATPase activity of platelet actomyosin showed pH optima at pH 5.8 and pH 9.5. The influence of the concentrations of calcium and ATP on the ATPase activity was studied and evidence was obtained that Ca-ATP was the substrate. Non-competitive inhibition was brought about by free ATP. Competitive inhibition was observed in the presence of ADP.

INTRODUCTION

Platelet actomyosin may be responsible for the contractile phenomena occurring during thrombin-induced viscous metamorphosis, pseudopodia-formation, release and clot retraction. The first to isolate and characterize the contractile proteins of human platelets were Bettex-Galland and Lüscher (1). Although many data have been reported about their solubility (1,2), ATPase activity (1,2,3,4), subcellular localization (3,4) and influence of haemostatically- active agents (4), relatively little attention has been paid to the structure of the protein-complex and to the kinetics of the ATPase activity. This paper reports the results of an analysis of the contractile complex by means of polyacrylamide gel electrophoresis and studies on the influence of calcium, ADP and ATP on the ATPase activity of platelet actomyosin.

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MATERIALS

Chemicals. ATP, ADP, aldolase and lactate dehydrogenase were obtained from Boehringer (Mannheim). Mersalyl was from Schuckhardt (München), bovine serum albumin from Poviet (Amsterdam) and Sepharose 4B from Pharmacia (Uppsala). Other reagents used, were of analytical grade purity.

Platelets. Platelet concentrates were obtained by centrifugation (320 x g for 15 min at 25°C) of about 4 l fresh normal human blood, collected in 0.15 volume of acid citrate dextrose solution. The platelets were harvested by centrifugation (3600 x g for 10 min at 4°C) and washed three times in 300 ml of 1% ammonium oxalate adjusted to pH 7.5 with NH_4OH, containing 0.1% EDTA. After washing the platelets were suspended in 25 ml 0.02 M TRIS-HCl buffer (pH 7.3) containing 0.6 M KCl (hereafter referred to as buffer A). The washed platelets were essentially free of leucocytes and erythrocytes (less than 1 : 10^5).

METHODS

ATPase assay. The standard assays for ATPase activity were performed by incubation at 25°C for 20 min of a mixture of 0.09 M Tris-HCl buffer (pH 9.0), 1 mM ATP, 1 mM $CaCl_2$, 0.03 M KCl and enzyme in a final volume of 2 ml. ATPase assays in the presence of $MgCl_2$ instead of $CaCl_2$ were performed at pH 7.0. For kinetic experiments the samples were incubated for 2 min at 37°C.

Enzyme activity was defined as nmoles P_i, formed per min per mg protein. Inorganic phosphate was determined by the method of Lecocq and Inesi (5).

Protein determination. The protein content was estimated by the method of Lowry et al (6) using crystalline bovine serum albumin as a standard.

Electrophoresis. Dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis was performed using a modification of the method of Weber and Osborn (7). Gels (7 x 0.5 cm) were prepared by addition of 1 ml acrylopropionitril to 50 ml of a 5% (w/v) solution of cyanogum-41 in 0.01 M phosphate buffer (pH 7.3) containing 0.5% ammoniumperoxidisulphate and 0.1% SDS (w/v). Electrophoresis was performed at a constant current of 8 mA per gel for about 2½ hour after a pre-electrophoresis period of 20 min in order to remove ammonium peroxidisulphate.

As reference proteins for the determination of the molecular weights were used: bovine serum albumin (MW 69.000), aldolase (MW 40.000) and lactate dehydrogenase (MW 35.000) (7). The gels were stained for two hours in a solution of 0.5% amido black in methanol, acetic acid, water (5:2:5). Destaining occurred in methanol, acetic acid, water (5:1:5) for about 36 hrs.

Antiserum. Antiserum to platelet actomyosin was obtained by immunization of rabbits into the foot pads and toe pads with 2 ml of an emulsified mixture of equal volumes of Freund adjuvant and purified platelet actomyosin solution (1 mg protein/ml). The animals were then boosted with injections of 1-2 mg protein at biweekly intervals for about 8 weeks. Thereafter they were bled by heart puncture. Serum was obtained by incubation at 37°C during 2 hours followed by centrifugation (30 min, 4.000 x g).

RESULTS

Isolation of platelet actomyosin. Platelet actomyosin was isolated from a platelet lysate by successive precipitation and gel filtration

TABLE I

Purification of Platelet Actomyosin

Step of purification	total protein (mg)	specific activity (nmoles/mg/min) calcium-dependent	magnesium-dependent
lysate	280	3,7	1,3
once precipitated	55	28	1,8
twice precipitated	25	43	1,9
sepharose 4B	8	44	2,3

6 g (wet weight) of platelets, suspended in 25 ml of buffer A were lysed for 20 hrs at 10°C in the presence of 2,5%-n-butanol, followed by centrifugation (25.000 x g for 60 min). Platelet actomyosin was precipitated from the supernatant, according to Grette (8). Gel filtration was performed on a 2,5 x 40 cm Sepharose 4 B column which was equilibrated and eluted with buffer A. Conditions of the ATPase assay were as outlined in Methods.

over a Sepharose 4B column. The degree of purification was determined by means of the calcium dependent ATPase activity at pH 9. An increase of the specific activity was obtained by two precipitation steps, whereas further precipitation or gel chromatography did not improve the specific activity (table 1). Magnesium-dependent ATPase activity had a low specific activity which only slightly increased after each purification step (table 1), suggesting that other enzymes with magnesium dependent ATPase activity were removed during purification.

Specific viscosity and sensitivity towards ATP. An average relative viscosity of 1.5 was found for solutions of platelet actomyosin in buffer A.

TABLE 2

The influence of ATP on the Viscosity of Platelet Actomyosin

Experiment	η_{rel}	η_{rel} ATP	ATP sensitivity	η_{spec}
1	1,53	1,31	57 %	0,15
2	1,45	1,25	67 %	0,16
3	1,38	1,22	58 %	0,12

The specific viscosity of platelet actomyosin in buffer A was calculated from relative viscosity determinations using a Cannon Fenske 50 viscosimeter at 25°C. The sensitivity towards ATP was calculated according to Portzehl (9)

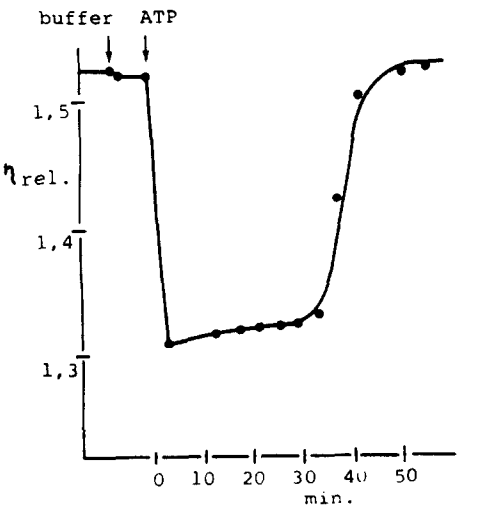

FIG. 1

Influence of ATP on the viscosity of platelet actomyosin. Protein concentration 2.8 mg/ml buffer A 1 mM ATP (2% by volume) added, temp. 25°C.

Addition of 0.1 ml of 50 mM ATP to 5 ml of a solution of platelet actomyosin resulted in a decrease in relative viscosity, which returned to the original value within 50 min (fig. 1). The average sensitivity towards ATP was 60 % and the specific viscosity 0.14. The results of three independent experiments are shown in table 2.

Immunological studies. The antiserum obtained by immunization of rabbits with purified platelet eactomyosin, was tested against whole platelet

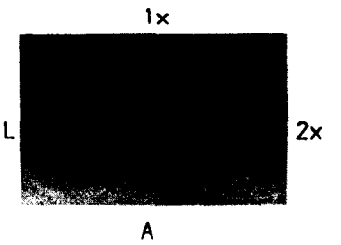

FIG. 2

Immunological characterization of different purification steps according to Ouchterlony (10). The agarose gels (1% w/v) were prepared with Tris-barbital-sodium-barbital, ionic strength 0.028, pH 8.8, containing 0.6 M KCl.
A : anti platelet actomyosin. L : platelet lysate.
1x : once precipitated actomyosin. 2x : twice precipitated actomyosin.

lysate, and against once and twice precipitated platelet actomyosin in an immunodiffusion experiment (fig. 2). Single precipitin lines showing a reaction of identity were observed against once and twice precipitated platelet actomyosin. Against the platelet lysate two precipitin lines were observed the lesser of which was probably caused by fibrinogen. The antiserum completely inhibited clot retraction of recalcified platelet-rich plasma at proper concentrations. These findings are in agreement with those of Nachman (3) and Cohen (11).

Dodecyl-sulphate polyacrylamide gel electrophoresis. Twice precipitated platelet actomyosin dialysed against 0.1% SDS and 1% β-mercapto-ethanol was separated by polyacrylamide gel electrophoresis in 4 main bands with calculated molecular weights of 220.000 (myosin), 175.000 (heavy meromyosin), 150.000 (myosin rod) and 45.000 (actin) (12,13). Two minor bands with molecular weights of about 100.000 and 30.000 may be identical with another cleavage product of the myosin molecule and platelet tropomyosin respectively (14) (fig. 3).

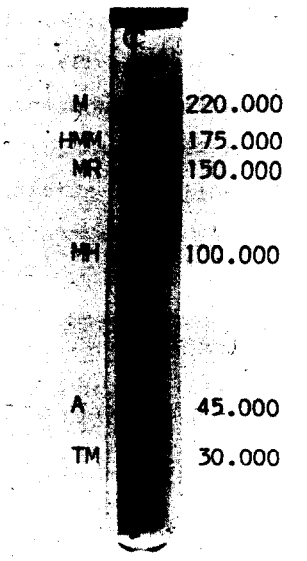

FIG. 3

SDS polyacrylamide gel electrophoresis of twice precipitated platelet actomyosin after preincubation in the presence of 1% β-mercapto-ethanol and 1% SDS; M = myosin, HMM = heavy meromyosin, MR = myosin rod, MH = myosin head, A = actin, TM = tropomyosin.

Characterization of the ATPase activity. Kinetic experiments were performed with enzyme preparations dialysed against chelex 100 in buffer A for 24 hours.

Inhibition. 10^{-4} M mersalyl completely inhibited the calcium dependent ATPase activity at both pH optima, suggesting that contaminating ATPases were not present (15).

pH optimum. The enzyme activity of platelet actomyosin has been studied in the pH range from 4 to 9.5. pH optima were demonstrated in the presence of calcium at pH 5.8 and at pH 9.5 (fig. 4). The pH optima in the presence of magnesium (fig. 5) were similar to those in the presence of calcium but the optimal activity was one-tenth. At pH 7 the ATPase activity in the presence of calcium was about 5% of the optimal value whereas magnesium induced a half-optimal value, which suggests the presence of a pH optimum for Mg dependent ATPase at pH 7.0 masked by calcium dependent ATPase activity caused by traces of calcium in the assay mixture.

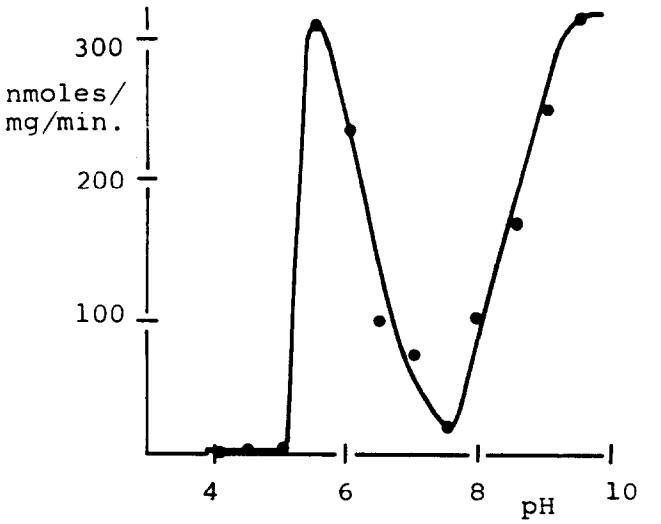

FIG. 4

ATPase activity of actomyosin as a function of pH in the presence of calcium. Assay mixture, 0.09 M Tris-Maleate buffer, 4 mM ATP, 0.03 M KCl and 10 mM $CaCl_2$, 0.15 mg protein/ml, 2 min incubation at 37°C.

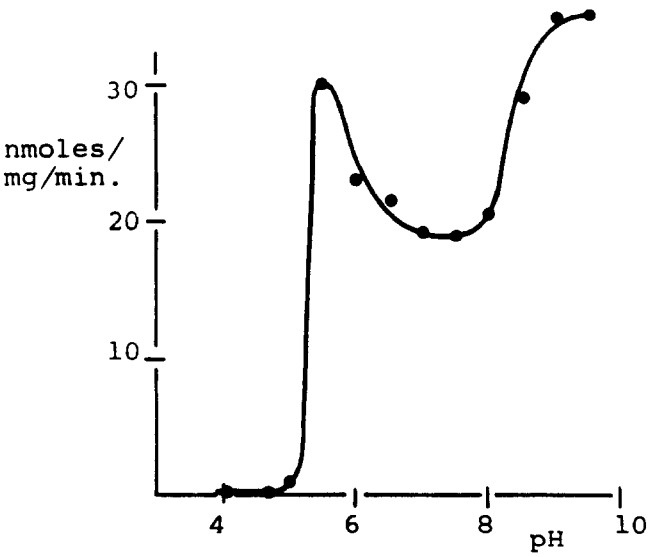

FIG. 5

ATPase activity of platelet actomyosin as a function of pH in the presence of magnesium. Assay mixture, 0.09 M Tris-Maleate buffer, 4 mM ATP, 0.03 M KCl, 10 mM $MgCl_2$, 0.15 mg protein/ml, 15 min incubation at 37°C.

Influence of ATP on calcium dependent ATPase activity. At various ATP concentration and at a constant calcium concentration (fig. 6), the enzyme

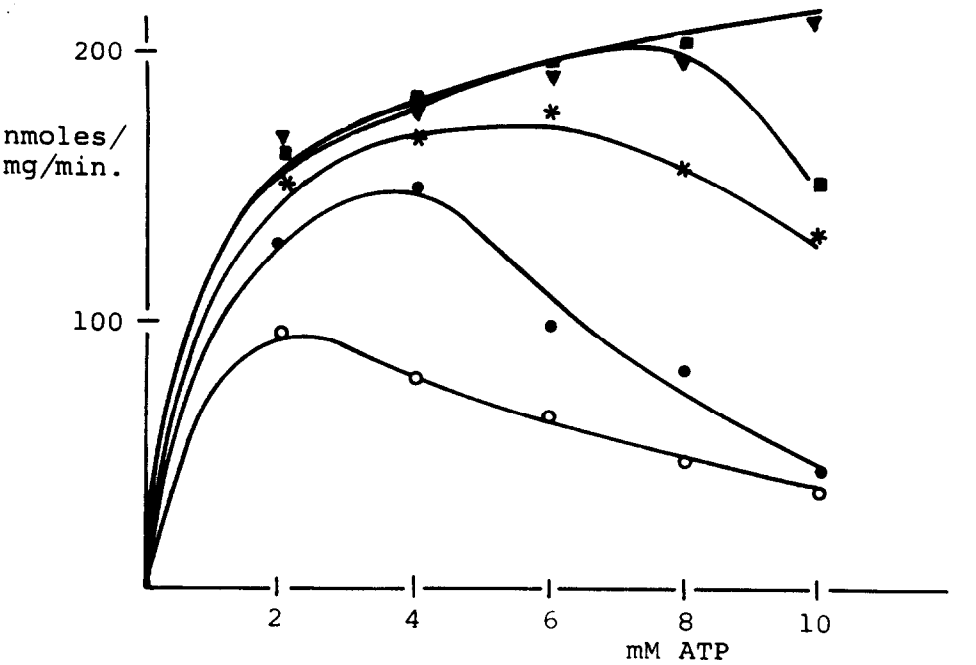

FIG. 6

ATPase activity as a function of ATP at different $CaCl_2$ at pH 9.
Further conditions: 0.09 M Tris-HCl buffer, 0.03 M KCl, 0.13 mg protein/ml.
2 min incubation at 37°C (o---o 2 mM $CaCl_2$, ●---● 4 mM $CaCl_2$,
x --- x 6 mM $CaCl_2$, ■---■ 8 mM $CaCl_2$, ▼---▼ 10 mM $CaCl_2$).

activity was inhibited at ATP concentrations exceeding those of calcium, which suggests that a complex of calcium and ATP is the substrate for platelet actomyosin.

For the determination of the K_m values the influence of free ATP was avoided by using a minimal excess of 4 mM of calcium (stability constant $Ca\text{-}ATP^{2-}$-32.000) (16). Directly after the purification procedure a K_m value of 0.5 mM was observed (fig. 7). This value changed in three days to 2 mM, while V_{max} increased from 250 to 300 nmoles P_i (mg) min.
The same values for K_m and V_{max} were obtained using higher calcium concentrations.

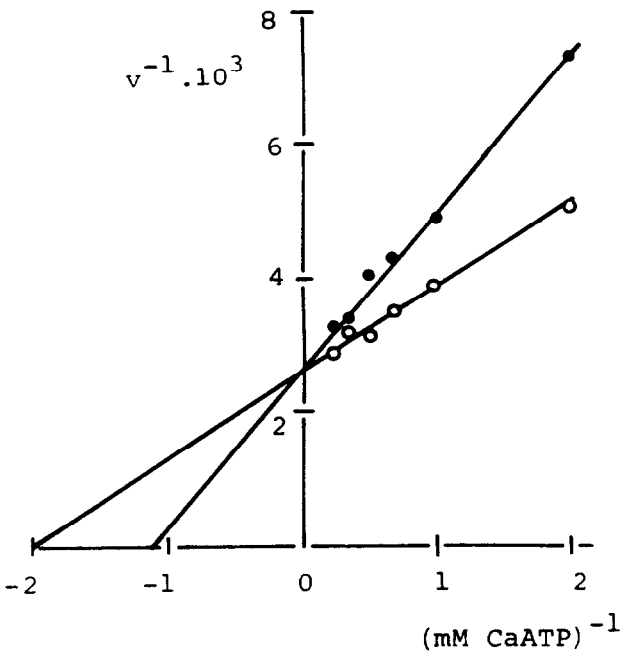

FIG. 7

The 1/V vs 1/Ca-ATP plot at pH 9 at 6 mM $CaCl_2$ in excess of ATP with and without 1.6 mM ADP. Further conditions: 0.09 M Tris-HCl buffer, 0.03 M KCl, 0.13 mg protein/ml. 2 min incubation at 37°C (o --- o 6 mM $CaCl_2$ excess, ● --- ● 6 mM $CaCl_2$, excess with 1.6 mM ADP).

The inhibitory effect of ATP on the enzyme activity was studied with an excess of 4 mM ATP as compared with calcium (fig. 8).

The K_m value remained constant, whereas the V_{max} shifted to a lower value. This non-competitive inhibition was observed directly after isolation of the enzyme as well as after three days. The influence of ADP on enzyme activity at an excess of 6 mM calcium is shown in figure 7.

No influence was observed on the V_{max} but the increase of the K_m suggested competitive inhibition.

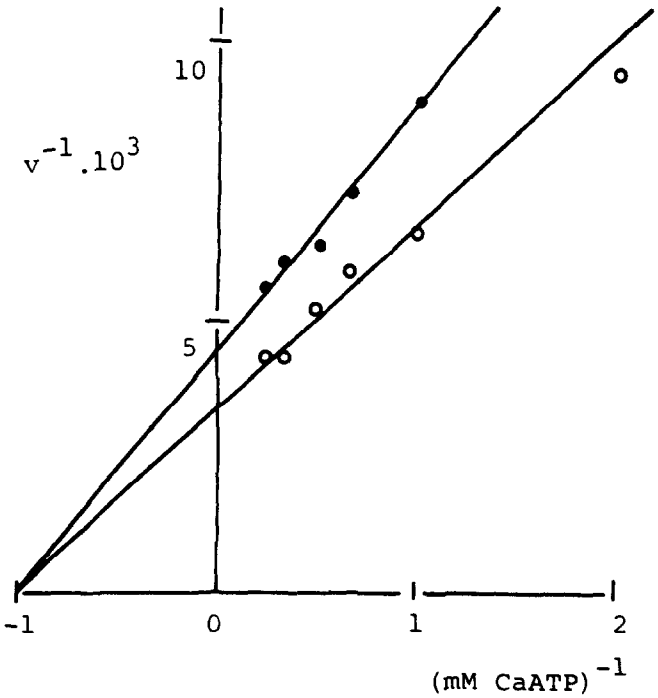

FIG. 8

The 1/V vs 1/ Ca-ATP plot at pH 9 with excess $CaCl_2$ or excess ATP.
Further conditions: 0.09 M Tris-HCl buffer, 0.03 M KCl,
0.13 mg protein/ml.
2 min incubation at 37°C (o --- o 6 mM $CaCl_2$ in excess of ATP,
● --- ● 4 mM ATP in excess of $CaCl_2$).

DISCUSSION

Platelet actomyosin or thrombostenin has many properties in common with contractile proteins of muscles. Physicochemical characterization of platelet actomyosin is therefore needed for a better understanding of platelet functions such as retraction and release in which contractile phenomena are involved (4).

In this investigation we tried to isolate the complete "contractile complex" including regulating proteins such as tropomyosin. This preparation was characterized by viscosity determination, superprecipitation studies (unpublished) and by ATPase assay. A rabbit antiserum against the purified preparation, inhibited clot retraction of recalcified platelet-rich plasma. The relatively high degree of purity of the enzyme preparation is illustrated by the presence, under experimental conditions, of only two precipitin lines

in an immunodiffusion experiment against platelet lysate (fig. 2), one of which is caused by antiplatelet actomyosin and the other probably by antifibrinogen.

Comparison of the specific activity of Ca-ATPase and Mg-ATPase (table 1) with those reported in the literature is difficult due to different assay conditions. Our results are generally in agreement with those found by others (1,2,3). The presence of free myosin fragments in the protein preparation may account for the relatively low value for ATP sensitivity (60%) as compared with that in other reports (60 - 90%). The specific viscosity (table 2) agrees well with previous results (1,2,3).

The observation that mersalyl, which is a rather specific inhibitor of contractile proteins of the actomyosin group (13), completely inhibited the Ca-ATPase activity, suggests that no contaminating enzymes with Ca-dependent ATPase activity were present.

The polyacrylamide gel electrophoresis experiments (fig. 3) showed that the enzyme preparation contained actin and myosin with molecular weights of about 45.000 and 220.000 respectively. The bands corresponding with molecular weights 150.000 and 100.000 are probably identical with the proteins characterized by Adelstein et al (13) as rod and head fragment of the myosin molecule. Another cleavage product of the myosin molecule may be the protein band with a moleculair weight of 175.000 since it corresponds with the molecular weight of heavy mermyosin, one of the fragments of muscle myosin after exposure to proteolytic enzymes. This proteolysis may have been initiated during purification by proteolytic enzymes present in platelets, or by thrombin (11,17). The low molecular weight fragments (light meromyosin and myosin head) may have been eliminated during purification because of their higher solubility at low ionic strength. The protein band corresponding with a molecular weight of about 30.000 is probably platelet tropomyosin (14).

Characterization of the ATPase activity of platelet actomyosin showed pH optima at pH 5.8 and pH 9.5 in the presence of calcium or magnesium (fig. 4 and 5). The specific activity at pH 7.5 is almost the same for magnesium as for calcium. At the optima however, the specific activity with calcium is ten times higher than that with magnesium. After pre-incubation of the enzyme preparation with EGTA, French (18) observed only one pH optimum in the presence of magnesium and two pH optima with calcium. This suggests that calcium ions remain bound to the enzyme and act on the pH profile if no chelating agent is used. This might mean that, in vivo also,

two pH optima exist of which the optimum at pH 5.8 probably has the more physiological significance (19,20).
The much higher specific activity found with Ca-ATP in comparison to Mg-ATP might be explained by the presence of a contaminating enzyme with Mg-ATPase activity as suggested by Abramowitz et al (21).
Another explanation might be that in our assay the majority of myosin is not bound to actin and as for smooth muscle, myosin is Ca-dependent and actomyosin is Mg-dependent (22).

Avena et al (22) reported that muscle myosin Ca-ATPase is inhibited at concentrations of ATP exceeding that of calcium by more than 1 mM. Our results indicate that platelet actomyosin is inhibited by ATP not complexed with calcium (fig. 6). This suggests that the Ca-ATP complex rather than free ATP is the substrate. By using excess calcium in comparison to ATP, all ATP was present as Ca-ATP complex. The K_m for the Ca-ATPase of 0.5 mM could thus be determined. The shift of the K_m from 0.5 to 2.0 mM Ca-ATP accompanied with a higher V_{max} in several days after storage may be caused by oxidation of SH-groups; however, the use of an SH reagent was not feasible as it causes splitting of the myosin molecule into subunits. Aggregation of actomyosin monomers during ageing might also be suggested as a cause of the shift in K_m.
Non-competitive inhibition of the Ca-ATPase was observed at an excess of ATP in comparison to calcium. This non-competitive inhibition suggests a non-catalytic binding site for ATP, which acts on the hydrolysis rate of the catalytic site.
The competitive inhibition which is observed when ADP is added to the assay mixture at an excess of calcium, can be explained as product inhibition.
The data given in this report increase the number of qualitative similarities reported between muscle and platelet actomyosin. However, whereas muscle actomyosin is strongly magnesium dependent, platelet actomyosin reaches the highest activities in the presence of calcium. The influence of tropomyosin and protein bound ions has not yet been determined in this respect. The amount of protein-bound tropomyosin and ions in the enzyme preparation probably depends on the purification procedure and may vary markedly in different reports.

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