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CONTROL OF PLATELET ACTOMYOSIN ACTIVITY: EFFECT OF ADP ON SUPERPRECIPITATION AND ATPase ACTIVITY OF HUMAN PLATELET ACTOMYOSIN*

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SUMMARY

1. Platelet actomyosin superprecipitation performed in 0.09 M to 0.12 M KCl showed a biphasic curve with clearing and turbidity increase after addition of Mg-ATP.

2. Addition of ADP produced inhibition of the duration and extent of the clearing phase (K_m of $4 \cdot 10^{-5}$ M), and activation of the superprecipitation phase (K_m of $1.96 \cdot 10^{-5}$ M).

3. The effect of ADP was observed with no significant increase of ATPase activity and was apparently specific for ADP and not for GDP, UDP, or IDP when superprecipitation was initiated only with Mg-ATP.

4. The effect of ADP was observed when superprecipitation occurred in the presence of EGTA.

These data suggest that ADP may form part of a control mechanism of platelet contractile activity.

INTRODUCTION

Blood platelets, through their actomyosin protein system, most likely mediate clot retraction, a phenomenon that contributes to the consolidation of the hemostatic plug [1]. Platelet aggregation, an earlier step, has been the subject of several studies [2–4] and although a number of hypotheses as to the mechanism of aggregation have been proposed [5], there is no agreement as to the extent of participation of the contractile protein system. This is due largely to a lack of detailed information concerning the nature of the molecular arrangement, localization, composition and control of the actomyosin protein complex. Recent findings suggest that a "relaxing protein complex", analogous to the troponin–tropomyosin system of muscle, is part of the platelet actomyosin complex [6–9]. This permits Ca^{2+} to participate in the regula-

Abbreviation: EGTA, ethylene glycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

* A preliminary report of this paper was presented at the Fed. Am. Soc. Exp. Med. Biol., Atlantic City, N.J., April 6th, 1973 [6].

tion of platelet contractile activity [8, 9]. In previous work it was suggested that ADP might play an important role in platelet actomyosin contractility [6–8]. In pursuing this observation we have examined the action of this nucleotide on superprecipitation and ATPase activity of platelet actomyosin [10]. ADP, which is also a known aggregating compound of platelets, was found to inhibit the dissociation of platelet actomyosin in the presence of Mg-ATP and to promote superprecipitation. These results lend support to the possible involvement of ADP in controlling the platelet contractile system and are consistent with the view that this contractile system participates in platelet aggregation.

EXPERIMENTAL PROCEDURE

Materials

Normal human blood was collected using the Latham Bowl plateletphoresis equipment, and platelets were prepared by differential centrifugation at 10 °C [11]. Platelet actomyosin was extracted from the equivalent of 5–6 units of blood from single donors. Muscle tissue was obtained fresh from rabbit back muscle [12]. All nucleotides used were obtained from Sigma Co. Crystallized bovine serum albumin was from Pentex Ill. Dithiothreitol was purchased from Calbiochem (San Diego, Calif.). All chemicals used were of reagent grade. Glassware was washed with either cleaning solution or 0.01 M EDTA; distilled water was passed through a mixed bed ion-exchange resin, and then glass distilled prior to use.

Methods

Protein extraction. Platelet buttons were washed once with 0.15 M NaCl, 0.1% EDTA, 0.1 mM dithiothreitol. The wash solution had been adjusted with Tris–acetate to pH 6.8. To extract actomyosin, the platelets were resuspended in 2 volumes of Weber–Edsall solution (0.6 M KCl, 0.01 M Na_2CO_3, 0.04 M $NaHCO_3$, pH 9.2) containing 0.1 mM dithiothreitol. The platelets were homogenized using the Parr Nitrogen pressure bomb at the pressure of 1200lb/inch^2 for 20 min. The resulting homogenate was further homogenized by 12 strokes in a Teflon–glass homogenizer powered by an electric motor set at the lowest setting of 150 RPM. The homogenate was left standing overnight at 4 °C, centrifuged at 60 000 $\times g$ for 60 min and the pellet discarded. Platelet actomyosin was then precipitated as described previously [11].

Muscle actomyosin was prepared from fresh muscles of rabbit by the procedure of Richards et al. [13]. Protein concentrations were measured by the method of Lowry et al. [14] and the standard was bovine serum albumin dissolved in 0.1 M or 0.6 M KCl, Tris 0.05 M, pH 7.2.

Determination of ATPase activity. ATPase activity of platelet proteins was determined by the release of inorganic phosphorus (P_i) from ATP according to Marsh [15] and adapted [16] to detect as little as 3 nmoles P_i. Each ml of final assay mixture contained 0.04 M imidazole buffer, pH 6.8, 0.06 M KCl, 0.5 mM ATP, 1 mM $MgCl_2$ or $CaCl_2$, and 0.1 mM ouabain. The amount of protein added to the final mixture was 0.1 mg actomyosin. Assays were performed on the proteins alone and after preincubation at room temperature for 5 min with 100 μM ADP. The reaction was stopped by the addition of 0.4 ml 20% trichloroacetic acid per ml mixture.

Blanks consisted of protein inactivated by trichloroacetic acid prior to addition of ATP. ATPase activity was estimated as the differences between P_i at a given time and P_i at zero time.

Superprecipitation. Superprecipitation was recorded by measuring absorbance of muscle actomyosin suspensions at 620 nm in a Gilford recording spectrophotometer. In a final volume of 1 ml prior to addition of ATP, 0.5 mg of platelet or muscle actomyosin was suspended in 0.9–0.12 or 0.15–0.16 M, KCl concentration buffered with 15–25 mM Tris–acetate, pH 6.8, respectively, to obtain a zero setting. Increasing concentrations of ADP were added to the protein prior to addition of Mg-ATP. At zero time 1 mM ATP plus 2 mM Mg^{2+} were added to the suspension, and the reaction was allowed to proceed at 25 °C. Clearing was observed as a decrease in absorbance whereas superprecipitation which followed caused increased absorbance [10].

RESULTS

KCl-dependence of superprecipitation

Platelet actomyosin superprecipitation occurring with 2 mM $MgCl_2$ and 1 mM ATP was optimal when KCl concentration was between 0.09 M and 0.12 M. For muscle actomyosin, under identical conditions, somewhat higher amounts of KCl were required (0.15–0.18 M). Superprecipitation was considered to be optimal when ATP-induced changes in absorbance began with a clearing phase, manifested by a drop in absorbance, lasting for 6–10 min, followed by an increase in turbidity with visible production of a precipitate. This precipitate eventually contracted to a small volume at the bottom of the cuvette. A curve was selected for "control" when conditions were set (KCl, temperature, protein concentration) to obtain a clearing phase that lasted the same time as that of superprecipitation (Fig. 1). Small variations in KCl concentration affected considerably the pattern of clearing and superprecipitation. At KCl concentrations less than 0.08 M, clearing was not observed and the onset of turbidity occurred immediately after addition of Mg-ATP. Above 0.12 M

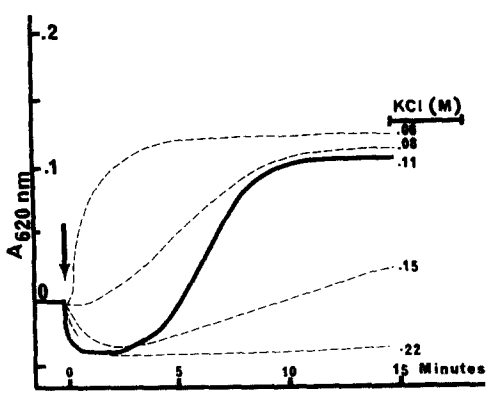

Fig. 1. Effect of KCl on the curve of superprecipitation of platelet actomyosin. The arrow indicates the time of addition of Mg-ATP. The solid line represents the biphasic curve that was used as control for most of the experiments. For further experimental detail refer to the text.

KCl, the clearing phase was prolonged with a delay in superprecipitation. Clearing was almost complete at KCl concentrations above 0.2 M.

Effect of KCl on the amount of protein involved in superprecipitation

Platelet actomyosin, dissolved in 0.6 M KCl–Tris buffer, was brought carefully to 0.09–0.12 M KCl by the stepwise addition of distilled water. Under these conditions the amount of protein that could be centrifuged and pelleted at low speed comprised 55% to 60% of the total protein in the reaction. Addition of Mg–ATP to the protein produced a drop in the optical density that was consistent with solubilization of the protein gel matrix as evidenced by the amount of protein that could be pelleted by centrifugation. Thus, the amount of protein that could be pelleted by low speed centrifugation decreased to approximately 45%. When superprecipitation was completed, 80–85% of the protein was found to be sedimented (Fig. 2). There was always a small amount of protein remaining in the supernatant. When the KCl concentration was below 0.09 M, the amount of protein in the pellet that was obtained at the end of the reaction (usually less than 5 min) was approximately similar to that measured at the 0.9–0.12 M range. When the amount of KCl was above 0.2 M the amount of protein pelleted was diminished as long as inhibition of superprecipitation persisted.

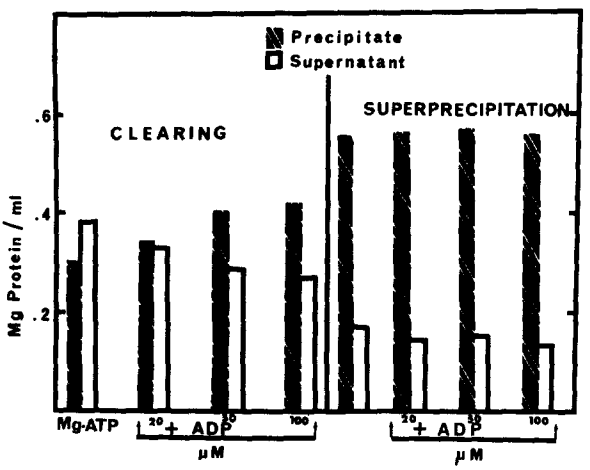

Fig. 2. Determination of the amount of protein during the clearing phase and during superprecipitation after the addition of Mg-ATP to platelet actomyosin. The protein was sedimented by low speed centrifugation. Clear bars indicate the amount of protein remaining in the supernatant after centrifugation. Area indicated by arrows shows the effect of different concentrations of ADP on the sedimentation of protein during both phases. Total protein was determined by the method of Lowry et al. [14].

Effect of ADP on the clearing phase

When the reaction mixture contained added ADP it was observed that with increasing ADP concentration both the duration and extent of the clearing phase decreased (Fig. 3). The duration of the clearing phase was inversely proportional to the concentration of ADP. At 200 μM ADP the clearing phase virtually disappeared. The amount of protein that could be sedimented 5 min after the initiation of the reaction by Mg-ATP was found to increase with increasing concentrations of ADP

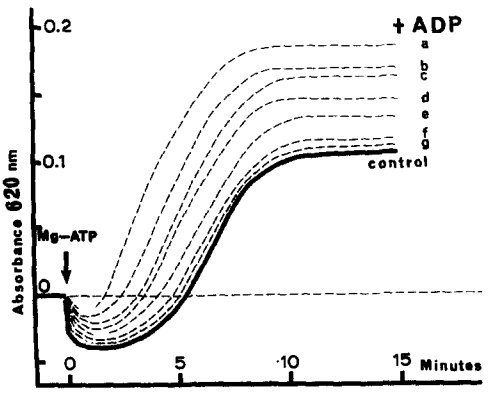

Fig. 3. Effect of different concentrations of ADP on the superprecipitation of platelet actomyosin initiated by the addition of Mg-ATP. Solid line indicates the control curve without addition of ADP. Broken lines indicate: a, 200 μM ADP; b, 100 μM ADP; c, 50 μM ADP; d, 20 μM ADP; e, 10 μM ADP; f, 5 μM ADP; g, 1 μM ADP.

(Fig. 2). With 100 μM ADP the amount of protein sedimented during the clearing phase was similar to that obtained before the addition of Mg-ATP where the sedimented proteins were in a loosely polydispersed gel form. In contrast, the sedimented proteins obtained after superprecipitation by Mg-ATP were present in a contracted form (syneresis) and were unchanged by ADP (Fig. 2).

Effect of ADP on superprecipitation

Increasing concentrations of ADP increased turbidity. Maximal turbidity was observed with 200 μM ADP (Fig. 3). The ADP effect is thus manifested by a shorter clearing phase and by an increase in turbidity during superprecipitation. 15 min after the addition of Mg-ATP, approximately 80% of the protein was pelleted by low speed centrifugation (Fig. 2). The increments in turbidity obtained at different ADP concentrations are shown in Fig. 4.

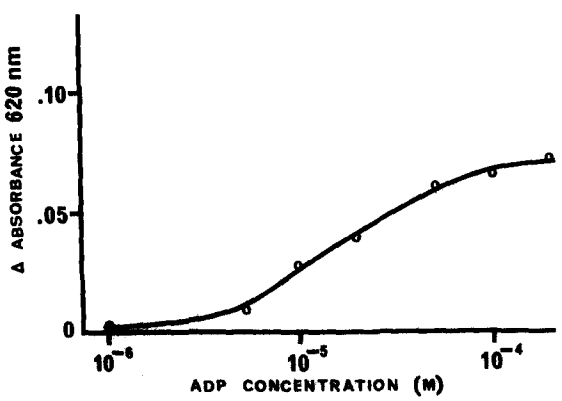

Fig. 4. Increments in absorbance at the end of superprecipitation produced by increasing concentrations of ADP used in the superprecipitation of platelet actomyosin by Mg-ATP.

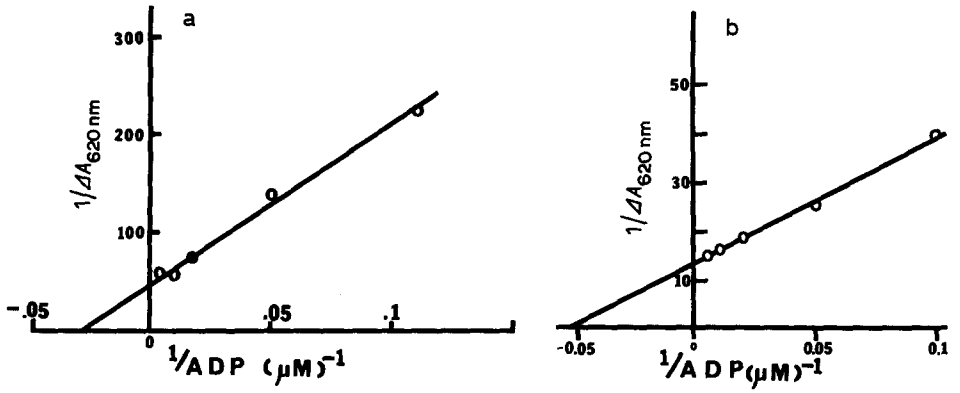

Fig. 5. a. Lineweaver–Burk plot of the effect of increasing concentrations of ADP on the turbidity of platelet actomyosin superprecipitation initiated after the addition of Mg-ATP. Data are those from Figs 3 and 4. Discussed in text. b. Lineweaver–Burk plot of the effect produced by increasing concentrations of ADP on the decrease in absorbance during the clearing phase initiated by the addition of Mg-ATP to platelet actomyosin. Data are those from Fig. 3. Discussed in text.

The apparent K_m for the ADP effect on turbidity was obtained by plotting the reciprocal of the increment of turbidity against that of ADP concentration (between 5 and 100 μM ADP) (Fig. 5a). The straight line obtained intercepted the ordinate giving an equivalent to V of 0.077 turbidity units per min. This line intercepted the abscissa giving a $-1/K_m$ value of 0.051 μM^{-1} which corresponds to an apparent K_m of $1.96 \cdot 10^{-5}$ M ADP.

The apparent K_m for ADP effect on the drop in absorbance (clearing) was obtained by plotting the reciprocal of the reduced absorbance against that of ADP concentration (between 5 and 100 μM ADP) (Fig. 5b). The straight line obtained intercepted the ordinate giving a V of 0.024 turbidity units per min. The line intercepted the abscissa giving a $-1/K_m$ value of 0.025 μM^{-1} which corresponds to an apparent K_m of $4 \cdot 10^{-5}$ M ADP.

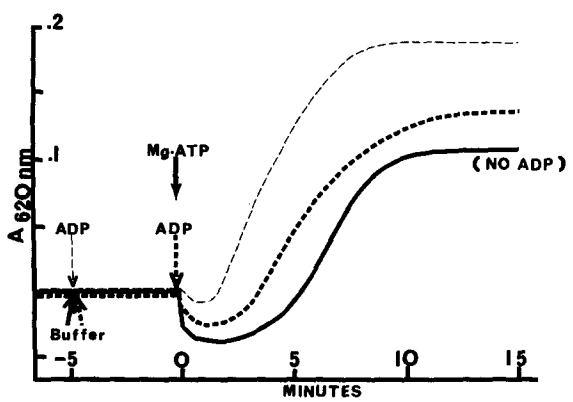

Fig. 6. Effect of ADP added 5 min before (– – –) and together (- - -) with Mg-ATP (———) on the clearing and superprecipitation of platelet actomyosin. Buffer is indicated as added to compensate for the volume when ADP was added together with Mg-ATP.

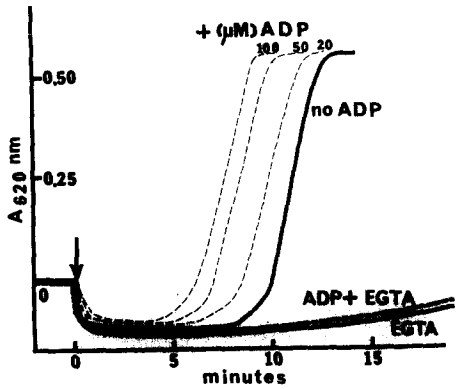

Fig. 7. Effect of different concentrations of ADP on the clearing and superprecipitation of muscle actomyosin initiated by Mg-ATP (arrow). EGTA was added alone and in combination with ADP to determine its effect on the reaction initiated by Mg-ATP. Discussed in text.

Preincubation of platelet actomyosin with ADP was found to be necessary for maximal effects. When ADP was added simultaneously with Mg-ATP a smaller effect was observed than when ADP was kept in the reaction for 5 min (Fig. 6) prior to the addition of Mg-ATP. Preincubation with ADP for more than 5 min did not increase further the extent of activation.

As with platelet actomyosin, increasing concentrations of ADP had the effect of shortening the clearing phase of muscle actomyosin (Fig. 7). It differed from platelet actomyosin because the extent of clearing, manifested by a drop in absorbance, did not vary and the turbidity level during superprecipitation was the same as that of the control without ADP.

Effect of Ca-ATP

The stimulatory effect of ADP was not seen when superprecipitation was initiated with Ca-ATP instead of Mg-ATP. Fig. 8 shows the pattern obtained when

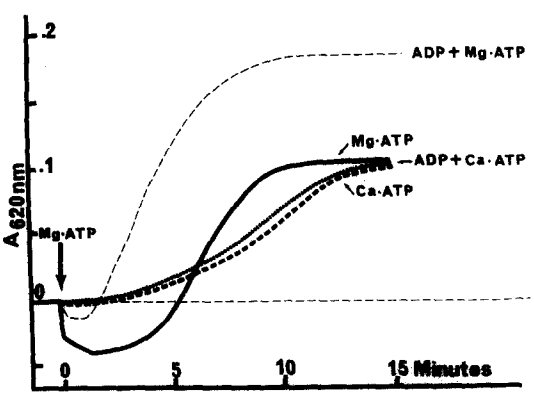

Fig. 8. Superprecipitation initiated by Ca-ATP. Effect of ADP (100 μM) on the clearing and superprecipitation initiated by Ca-ATP. The patterns obtained from Fig. 6 are shown for comparison.

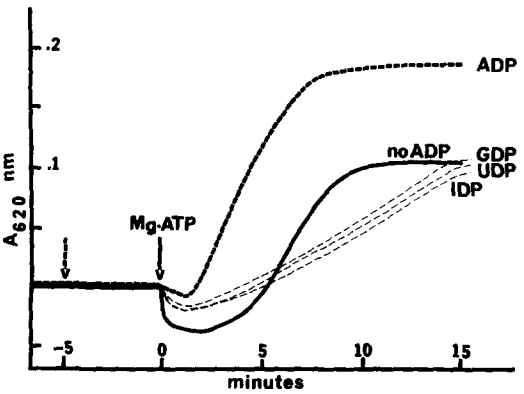

Fig. 9. Effect of GDP (100 μM), UDP (100 μM), IDP (110 μM), and ADP (100 μM), on the superprecipitation of platelet actomyosin initiated by Mg-ATP.

2 mM $CaCl_2$–1 mM ATP were used. No clearing phase with Ca^{2+} could be obtained. When different concentrations of Ca-ATP (0.1–2 mM) were used the curve varied from complete absence of superprecipitation to that obtained with 2 mM Ca^{2+}.

The clearing phase could not be obtained by varying the concentrations of KCl. 100 μM ADP did not alter superprecipitation in the presence of Ca-ATP.

Effect of other nucleotides

The specificity of the ADP effect on platelet actomyosin superprecipitation was tested. GDP, UDP and IDP were used in conjunction with Mg-ATP (Fig. 9). The use of nucleotides other than ADP seemed to have altered the pattern of the Mg-ATP effect. The clearing phase was depressed.

In comparison to ADP, the nucleotides GDP, UDP, and IDP showed no enhancement of turbidity during superprecipitation. To establish whether the use of heterologous nucleotides (i.e. ATP and GDP) was responsible for the failure to

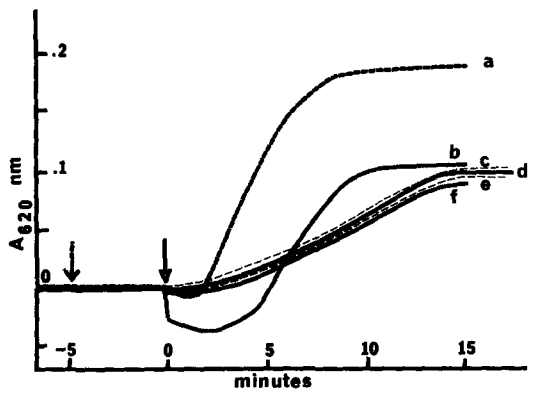

Fig. 10. Effect of ADP (a), GDP (c), and UDP (e), at 100 μM each, on the superprecipitation of platelet actomyosin initiated by Mg-ATP (b) and (a), Mg-GTP (d) and (c) and Mg-UTP (f) and (e), respectively. Solid arrow indicates addition of Mg-ATP, or Mg-GTP or Mg-UTP. Broken arrow indicates preincubation for 5 min with either ADP, GDP or UDP.

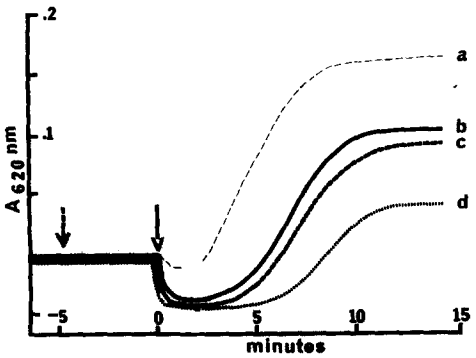

Fig. 11. Effect of EGTA on the clearing and superprecipitation of platelet actomyosin initiated by Mg-ATP; (a) effect of ADP without EGTA on the reaction initiated by Mg-ATP (solid arrow); (b) Mg-ATP control; (c) effect of ADP and EGTA preincubated for 5 min before addition of Mg-ATP; (d) effect of EGTA on the superprecipitation initiated by Mg-ATP. Discussed in text.

reproduce the typical sigmoid curve, GDP was used in combination with Mg^{2+}-GTP and similarly UDP with Mg^{2+}-UTP. In both instances clearing could not be produced and the corresponding nucleotide diphosphate did not enhance turbidity values when compared with Mg^{2+}-ATP (Fig. 10).

Effect of EGTA

When EGTA (0.25 mM) was added to platelet actomyosin the clearing phase was prolonged and the turbidity decreased. ADP in the presence of EGTA produced no essential change in the absorbance of the clearing phase but was capable of shortening it. In addition, ADP enhanced the turbidity during superprecipitation (Fig. 11).

Effect of ADP and EGTA on ATPase activity

ATPase determination on platelet and muscle actomyosin aliquots undergoing superprecipitation in the presence of ADP failed to show a slight increase of P_i

TABLE I

EFFECT OF ADP AND EGTA ON ATPase ACTIVITY OF PLATELET AND MUSCLE ACTOMYOSIN DURING SUPERPRECIPITATION BY Mg-ATP

Reaction mixtures are described in the text.

Time (min)	Platelet actomyosin μmoles of P_i per mg protein			Muscle actomyosin μmoles of P_i per mg protein		
	−ADP	+ADP	+EGTA	−ADP	+ADP	+EGTA
1	0.03	0.04	0.01	0.65	0.75	0.12
2	0.07	0.08	0.03	1.10	1.15	0.14
3	0.09	0.11	0.05	2.05	2.20	0.21
4	0.12	0.14	0.07	3.40	3.45	0.27
5	0.15	0.17	0.09	4.20	4.25	0.32
10	0.23	0.28	0.13	7.55	7.95	0.59
15	0.32	0.36	0.17	10.66	10.90	0.77

release over that of controls without added ADP (Table I). When the effect of EGTA on ATPase activity was measured, it was found that the ATPase activity was reduced in half with platelet actomyosin while with muscle actomyosin the ATPase activity was reduced 10–15 times from that of control without EGTA.

DISCUSSION

At present, little is known of the mechanisms which control the contractile activity of platelet actomyosin [7, 8]. Previous findings have demonstrated that the ATPase activity of platelet actomyosin responds to Ca^{2+} in a manner similar to cardiac and muscle actomyosin [7, 8]. This control mechanism was also manifested in isolated platelet actomyosin by an increase in Mg^{2+}-activated ATPase activity when the ionized Ca^{2+} concentration was raised within to a range between 10^{-7}–10^{-5} M [8]. In the present study the conditions for platelet actomyosin superprecipitation were examined and the possibility that products of ATP hydrolysis could also control contractile activity was studied.

One parameter of interest was the apparent lack of parallelism between ATPase activity and the time of onset and intensity of superprecipitation in platelet actomyosin. In platelets, superprecipitation was obtained and completed when less than 1% of added ATP was hydrolyzed [8].

It has been reported that muscle actomyosin, by virtue of its higher ATPase activity, quickly reduces the amount of free ATP in the medium with concomitant production of superprecipitation [17], after which ATPase activity increases [17, 18]. Platelet actomyosin has approx. 10 times less ATPase activity than muscle actomyosin, yet superprecipitation occurs shortly after the addition of ATP. That the turbidity increase represented true superprecipitation could be shown by measurement of the volume of the protein pellets obtained with and without addition of Mg-ATP. Although the amount of total proteins sedimented after adding Mg-ATP was higher than without Mg-ATP, the volume of the contracted precipitate after Mg-ATP (syneresis) was considerably smaller than that obtained by sedimentation without Mg-ATP [19]. The concentration of KCl present in the reaction mixture was of importance in that below and above a relatively small range (0.09–0.12 M) rapid formation of a superprecipitate or complete clearing was observed, respectively.

The hypothesis as to whether factors other than the lowering of free ATP concentration were responsible for superprecipitation, i.e., the appearance of ADP, was tested. The addition of exogenous ADP clearly accelerated the time and degree of superprecipitation, with a simultaneous reduction of the extent of clearing. Only ADP and none of the other nucleotide diphosphates was effective in activating superprecipitation. Half maximal activation of turbidity was found to be 20 μM for ADP and the concentration of ADP that produces half maximal inhibition of the clearing phase was found to be approx. 40 μM (Fig. 11).

One interesting difference between platelet and muscle actomyosin is the amount of protein involved in the process of superprecipitation. When superprecipitation of muscle actomyosin was determined for purposes of comparison, it was accelerated by ADP (Fig. 7), but the extent of clearing and turbidity, and the amounts of protein involved in the reactions were always the same for different ADP concentrations. With platelet actomyosin, similar to muscle, the amount of protein partici-

pating in the reaction was the same, but the degree of turbidity achieved varied with different concentrations of ADP used. It is possible than that ADP alters the degree of aggregability (cross linking) of the superprecipitated platelet actomyosin.

With the addition of 0.25 mM EGTA, superprecipitation of platelet actomyosin was inhibited approx. 50%, while at the same time the clearing phase was prolonged by approx. 50% (5–7.5 min). Furthermore, with EGTA the ATPase activity of platelet actomyosin after addition of Mg-ATP was also reduced approx. in half. These findings suggested that Ca^{2+} partially regulates, under these conditions, the platelet actomyosin contractile activity. The fact that ADP can still promote superprecipitation in the presence of EGTA suggests that ADP control is independent of that exerted by Ca^{2+}.

The finding that, among the nucleotide diphosphates, only ADP shows this effect may be of significance because ADP is present in relatively large amounts in the platelet cytoplasm [1]. ADP is known to be one of the principal mediators of platelet aggregation, a property that leads eventually to larger platelet aggregates that under appropriate conditions is followed by contraction of the aggregates. Furthermore, the possible localization of platelet actomyosin in the outer membrane [5] could make this unusually localized actomyosin the target of an early effect by ADP that would facilitate the participation of cytoplasmic actomyosin in the overall reaction of platelet contraction [20].

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