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ACTION OF ADP AND ETHANOL ON MUSCLE PROTEINS *IN VITRO*

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Abstract—The effects of ADP and ethanol on Ca^{2+} binding of troponin and the superprecipitation of actomyosin were studied. Ca^{2+} binding of troponin–tropomyosin complex bound to polystyrene particles (Lytron) was increased by ADP, and this increase was inhibited by ethanol. However, Ca^{2+} binding of the complex as measured by equilibrium dialysis and by the chelex resin method was not influenced by either ADP or ADP plus ethanol. Ca^{2+} binding of the thin filament, myosin-ghost myofibrils and myofibrils was also not inhibited. Superprecipitation of actomyosin was augmented in the presence of ADP, and the enhancement was inhibited by ethanol. However, this effect of ADP or ethanol was not observed in the presence of an inhibitor of myokinase, p^1, p^5-di (adenosine-5′) pentaphosphate (Ap_5A). In the presence of Ap_5A, superprecipitation of actomyosin was enhanced when small amounts of ATP (10 μM) and ADP (100 μM) were added 5 min prior to the addition of 2 mM ATP to start the reaction. The enhancement of superprecipitation of actomyosin by ADP may be caused by nuclei produced by a low concentration of ATP which is produced from ADP by contaminating myokinase activity. These data suggest that ADP and ethanol influence Ca^{2+} binding of the troponin–tropomyosin complex bound to a solid phase, but their effects on superprecipitation may not necessarily reflect muscle contraction *in vivo*.

Acute ethanol ingestion decreases the contractility of heart and skeletal muscles [1–4]. It has also been demonstrated that chronic ethanol ingestion leads to striking ultrastructural changes in skeletal muscle [5]. Actomyosin prepared from the skeletal muscle of baboons and volunteers fed ethanol demonstrated decreased ATPase activity and decreased calcium sensitivity of the ATPase [5]. *In vitro* contractility of isolated actomyosin as measured by superprecipitation is inhibited by ethanol, and actomyosin from animals and volunteers fed ethanol chronically displayed impaired superprecipitation *in vitro* in the absence of ethanol [6]. Based upon the effect of ethanol and ADP on superprecipitation and the ATPase activity of actomyosin, a model has been proposed to explain the effect of ethanol on the regulated actomyosin system [5, 7]. Since superprecipitation, the ATPase activity of actomyosin, and the Ca^{2+} binding of the troponin–tropomyosin complex bound to Lytron particles were enhanced by ADP and inhibited by ethanol, the changes produced by ADP and ethanol were thought to be related to a change in Ca^{2+} binding by troponin.

In this paper, we present studies of the effect of ADP and ethanol on various components of the contractile system. The purified muscle proteins, troponin, tropomyosin, actin and their mixtures, as well as myofibrils, were studied by measuring different variables, including Ca^{2+} binding, ATPase activity, and superprecipitation. Based upon these results, the previous model is reevaluated.

MATERIALS AND METHODS

Chemicals. Tetramethyl murexide was purchased from Calbiochem-Behring Diagnosis (San Diego, CA). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Lytron 615 (polystyrene particles) was purchased from the Monsanto Chemical Co. (Springfield, MA).

Preparation. The proteins used in this study were prepared from rabbit skeletal muscle. Troponin and tropomyosin were isolated by the method of Ebashi *et al.* [8]. Troponin was purified by DEAE cellulose column chromatography [9]. Tropomyosin was purified by isoelectric precipitation and ammonium sulfate fractionation. Actin was isolated and purified by the method of Spudich and Watt [10]. Myosin B was obtained by the method of Szent-Gyorgi [11], and desensitized actomyosin was obtained by washing myosin B with 2 mM bicarbonate ten times [12]. Myofibrils were prepared by the method of Etlinger and Fischman [13]. To prepare myosin ghost myofibrils, myosin was removed from the myofibrils by washing with Guba–Straub solution (K^+ concentration: 0.45 M) containing 2 mM ATP [14].

Ca^{2+}–EGTA‖ buffer. For the Ca^{2+}-binding experiments, except for the chelex resin method, the desired concentrations of free Ca^{2+} were obtained

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‖ Abbreviations: EGTA, ethyleneglycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Ap_5A, p^1,p^5-di (adenosine-5′) pentaphosphate; DTT, dithiothreitol; and HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

by using a Ca^{2+}–EGTA buffer (concentration of EGTA = 0.1 mM). The concentration of contaminating calcium in protein samples was determined by atomic absorption spectroscopy. The contaminating calcium in KCl–HEPES solution was determined by the tetramethyl murexide method [15]. The solvent conditions used for Ca^{2+}-binding measurements were 100 mM KCl, 20 mM HEPES (pH 7.4) and 1 mM DDT, in the presence or absence of 2 mM Mg^{2+} at 25°.

Ca^{2+} binding of protein–Lytron particles. Ca^{2+} binding by protein adsorbed on Lytron particles was assayed essentially according to Puszkin and Rubin [7]. Lytron particles were sedimented from the original stock suspension at 20,000 *g* for 1 hr, washed once with water, and resuspended in the respective reaction medium [16]. Particles were coated with proteins as follows. The Lytron particle suspension and the troponin solution were mixed and incubated for 10 min at 4°, and the mixture was centrifuged at 10,000 *g* for 10 min. The pellet was repeatedly washed in the same buffer solution until no protein was detected in the supernatant fraction. The amount of troponin adsorbed on Lytron particles was calculated by subtracting the amount of protein in the supernatant fraction from the total amount of protein. Troponin–tropomyosin coated Lytron particles were prepared by adding troponin to tropomyosin-coated Lytron particles. Ca^{2+} binding was measured by using radioisotope ^{45}Ca. Troponin–Lytron or troponin–tropomyosin–Lytron particles were first washed in a buffer solution containing 0.1 mM EGTA (without Ca^{2+}), to remove Ca^{2+} bound to troponin. The washed particles were resuspended in the reaction medium containing ^{45}Ca and incubated with shaking for 30 min at 25°. The particles were sedimented by centrifugation, and the amount of Ca^{2+} bound to troponin was estimated from the decrease in radioactivity of the supernatant fraction.

Ca^{2+} binding measured by the chelex method. For this measurement, Ca^{2+}-free troponin was prepared by the method of Iida [17]. Troponin was added to 10 mM HCl, and dialyzed against 5 mM HCl overnight. It was dialyzed against water several times. According to Iida, the protein prepared by this method contains less than 10^{-8} M Ca^{2+}. We tested our samples by atomic absorption spectroscopy and found the content of Ca^{2+} to be negligible. The chelex resin was washed extensively with HCl and then rinsed with water. The resin was then equilibrated with the buffer solution to be used. The resin suspension was mixed with a buffer containing ^{45}Ca in the absence and presence of troponin. After 30 min of incubation, with shaking at 25°, the chelex resin was sedimented by centrifugation at 2000 *g* for 5 min. In this method, Ca^{2+}–chelex suspension serves as the Ca^{2+} buffer system. The data were analyzed to calculate the Ca^{2+} bound to troponin according to the method of Fuchs and Briggs [18].

Ca^{2+} binding measured by equilibrium dialysis. The troponin solution was first dialyzed against a buffer containing 0.1 mM EGTA to remove bound Ca^{2+}. It was then dialyzed against a Ca^{2+}–EGTA buffer containing ^{45}Ca. The dialysis was carried out overnight at 25°. The amount of Ca^{2+} bound to troponin was estimated from the difference in radioactivity between the troponin solution and the dialysate.

Ca^{2+} binding measured by millipore filtration and centrifugation. These methods were used for the determination of Ca^{2+} bound to actomyosin in the absence and presence of ATP. The actomyosin sample was suspended in a solution containing 20 mM Tris–maleate (pH 6.8), 60 mM KCl, 2 mM $MgCl_2$ and ^{45}Ca at 25°, and was incubated for 30 min in the cuvette of a spectrophotometer. ATP (2 mM) was added and superprecipitation was followed spectrophotometrically [19]. When superprecipitation was completed, the actomyosin precipitate was separated either by centrifugation (8000 *g* for 10 min) or by filtration through a millipore filter (0.45 μm pore size).

In the centrifugation method, Ca^{2+} bound to the precipitated actomyosin was estimated from the decrease in radioactivity in the supernatant fraction. In the millipore filtration method, the protein trapped on a millipore filter was washed with a buffer which does not contain Ca^{2+}. The filter was then placed in a scintillation vial, scintillation counting solution was added, and the radioactivity was measured.

Superprecipitation. Superprecipitation was determined by measuring the turbidity change at 500 nm. The effects of ADP and/or ethanol on superprecipitation were studied by measuring the time required for the optical density to reach half of its maximum change ($\tau_{1/2}$) [19]. The solvent conditions for superprecipitation were as follows: 20 mM Tris–maleate (pH 6.8), 2 mM ATP and various concentrations of KCl and $MgCl_2$. The temperature was 25°. In this paper, the reciprocal of $\tau_{1/2}$ is used to show the contractility of actomyosin *in vitro*. The effect of ADP or ethanol is shown in Figs. 4–6 as the ratio of contractility in the presence of the compound to that in its absence.

ATPase activity. ATPase activity was determined by measuring liberated inorganic phosphorus (P_i), according to Ohnishi and Gall [20]. The solvent was the same as in the study of superprecipitation.

RESULTS

Ca^{2+} binding measured by the Lytron particle method. Lytron particles were first saturated with tropomyosin and subsequently saturated with troponin (troponin/tropmyosin = 0.7). Figure 1 shows Ca^{2+} binding by troponin–tropomyosin in the absence and presence of 2 mM $MgCl_2$. In the absence of ADP, the ratio of Ca^{2+}/troponin did not reach the theoretical value of 4 [21], even though the Ca^{2+} concentration was increased to 2.0×10^{-5} M. In this case, the binding curves were not influenced by the addition of ethanol. Ca^{2+} binding was enhanced by 100 μM ADP (Fig. 1). This increase was partially inhibited by 0.5% ethanol. These effects were observed in the presence or absence of 2 mM $MgCl_2$.

Ca^{2+} binding was also studied using Lytron particles coated with a single layer of troponin (Fig. 1). The amount of bound Ca^{2+} was less than that observed for tropomyosin–troponin–Lytron particles, and ADP and ethanol were without effect.

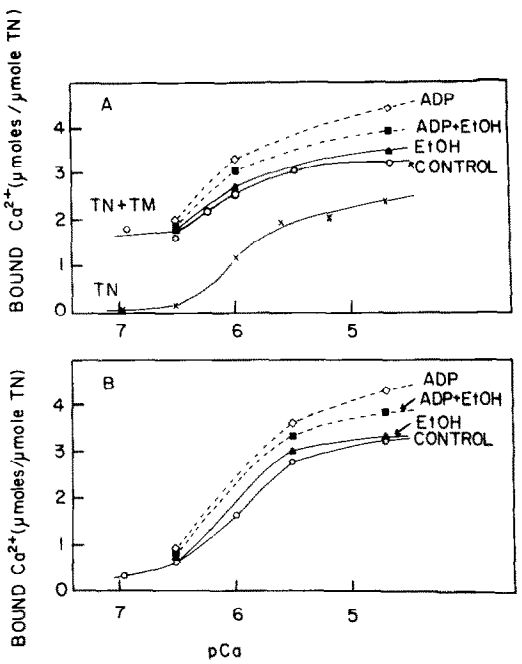

Fig. 1. Effects of ethanol and ADP on Ca^{2+} binding by troponin–tropomyosin–Lytron particles and troponin Lytron particles. Lytron particles were coated first with tropomyosin and then with troponin (all symbols except x), or only with troponin (x). Tropomyosin, 0.86 mg, was adsorbed on 30 mg of Lytron particles; the ratio of troponin to tropomyosin was 0.7. The effects of ethanol and ADP on Ca^{2+} binding were measured (A) in the absence of $MgCl_2$ and (B) in the presence of 2 mM $MgCl_2$. The symbols are: control (○—○), 0.5% ethanol (▲—▲), 100 μM ADP (◇---◇) and 0.5% ethanol plus 100 μM ADP (■---■). The concentration of free Ca^{2+} was obtained by using a Ca^{2+}–EGTA buffer (concentration of EGTA = 0.1 mM). The other experimental conditions included: 100 mM KCl, 20 mM HEPES (pH 7.4) and 1 mM DTT, at 25°.

Table 1 shows the effects of nucleotides on Ca^{2+} binding by troponin–tropomyosin–Lytron particles. An increase in Ca^{2+} binding was observed only with ADP. However, the reduction in Ca^{2+} binding by ethanol was found for all nucleotides.

Ca^{2+} binding measured by the chelex resin method. Figure 2 shows the Ca^{2+} binding curves of troponin using the chelex resin method. Similar to the results obtained by the Lytron particle method, neither ethanol nor ADP changed Ca^{2+} binding by troponin. Ca^{2+} binding by a mixture of troponin and tropomyosin was also studied in the absence of Mg^{2+} with the chelex resin method (Table 2). ADP and ethanol had no effect on Ca^{2+} binding.

Ca^{2+} binding measured by equilibrium dialysis. Figure 3 illustrates Ca^{2+} binding by troponin determined by the equilibrium dialysis method. The binding properties are comparable to those demonstrated by Potter and Gergely [21]. There was no effect of ADP or ethanol, in the presence or absence of Mg^{2+}.

Ca^{2+} binding by troponin in various complexes was also determined in the absence of Mg^{2+} by equilibrium dialysis (Table 2). No effect of ADP or ethanol was obtained with tropomyosin–troponin, actin–tropomyosin–troponin, actomyosin, myosin ghost myofibrils and myofibrils.

Ca^{2+} binding by actomyosin in the presence of ATP. Millipore filtration and centrifugation were used to study the Ca^{2+} binding of actomyosin in the presence of ATP (Table 3). Both methods yielded similar results. Although the amount of Ca^{2+} bound to actomyosin was not changed by ADP or ethanol in the absence of ATP, it was reduced by ADP or ethanol in the presence of ATP. When both ADP and ethanol were added, Ca^{2+} bound to actomyosin increased, compared to the amount bound in the presence of either ADP or ethanol alone.

Effects of ADP and ethanol on actomyosin. The addition of ADP shortened the time required for superprecipitation, while the addition of ethanol inhibited that effect (Fig. 4).

The ATPase activity of actomyosin was measured under the same conditions as in the study of superprecipitation (Fig. 5). However, little effect of ADP on actomyosin ATPase was found, compared with that on superprecipitation.

Effects of a myokinase inhibitor. Superprecipitation and ATPase activity were measured in the presence of the myokinase inhibitor Ap_5A (Fig. 5). Ap_5A alone had no effect on superprecipitation or ATPase activity in the absence of ADP. However, in the presence of ADP, Ap_5A decreased the velocity of the superprecipitation (Fig. 5). The effect on ATPase activity was much less than that on superprecipitation.

Effect of a small amount of ATP on actomyosin activity. In the presence of myokinase, ATP and AMP are formed from ADP:

$$2\,ADP \rightleftharpoons ATP + AMP.$$

We, therefore, inquired whether the observed ADP effect was caused either by ATP or AMP produced

Table 1. Effects of various nucleotides and ethanol on Ca^{2+} binding by troponin–tropomyosin–Lytron particles*

Nucleotide	Bound Ca^{2+} (μmoles/μmole troponin)	
	No ethanol	0.5% Ethanol
None	2.89 ± 0.13	2.80 ± 0.14
ADP	3.30 ± 0.15	3.08 ± 0.12
ATP	2.87 ± 0.12	2.55 ± 0.10
AMP	2.81 ± 0.17	2.45 ± 0.13

* The concentration of free Ca^{2+} was 1.0×10^{-6} M. Other conditions were the same as those in Fig. 1B. Values are means ± S.D. obtained from four measurements.

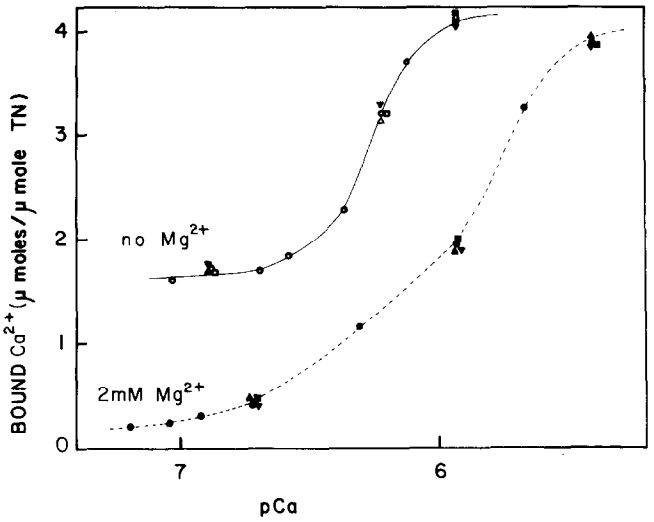

Fig. 2. Effects of ethanol and ADP on Ca^{2+} binding by troponin measured by the chelex resin method in the absence and presence of 2 mM Mg^{2+}. The concentration of troponin was 1 mg/ml. Experimental conditions were the same as shown in Fig. 1, but Ca^{2+}–EGTA buffer was not used. Open symbols (solid line) represent the absence of Mg^{2+} and closed symbols (dashed line) the presence of 2 mM Mg^{2+}; control (○, ●), 0.5% ethanol (△, ▲), 100 μM ADP (▽, ▼) and 0.5% ethanol plus 100 μM ADP (□, ■).

by this reaction. We added a small amount of ATP (10 μM) 5 min prior to the start of the measurement in the presence of Ap_5A. We then added 2 mM ATP and measured the ATPase activity and superprecipitation (Fig. 6).

As shown in the figure, a small amount of ATP did not change the velocity of superprecipitation in the absence of ADP. However, the velocity of superprecipitation increased with an increasing concentration of ADP. Ethanol inhibited the increase in the velocity produced by ADP. In the absence of ADP, no change was produced by the addition of ethanol.

Figure 7 shows the effect of a small amount of ATP added 5 min prior to the experiment, as measured by the ATPase activity in the presence of Ap_5A. In the absence of ADP, the ATPase activity decreased with an increase of a small amount of ATP (10–50 μM), which was added prior to the addition of 2 mM ATP. However, ADP (0.1 mM) reversed this effect. We have also tested the effect of AMP, but found that it did not show a significant effect at 0.1 mM (data not shown).

DISCUSSION

It has been shown that acute ethanol ingestion decreases the contractility of muscle [1–4], but the molecular mechanism of this action has not been elucidated. To study the effect of ethanol on con-

Table 2. Effects of ethanol and ADP on Ca^{2+} binding by troponin in various systems*

Preparation	Method	Ca^{2+} binding			
		Control	Ethanol (0.5%)	ADP (0.1 mM)	ADP + ethanol
Troponin + tropomyosin	(†)	3.20 ± 0.10	3.12 ± 0.12	3.25 ± 0.14	3.17 ± 0.11
(μmoles Ca^{2+}/μmole troponin)	(‡)	2.91 ± 0.12	2.74 ± 0.11	2.85 ± 0.09	2.98 ± 0.14
	(§)	3.76 ± 0.12	3.73 ± 0.14	3.83 ± 0.16	3.66 ± 0.15
F-actin + tropomyosin + troponin (μmoles Ca^{2+}/μmole troponin)	(‡)	2.49 ± 0.09	2.51 ± 0.12	2.53 ± 0.10	2.59 ± 0.12
Actomyosin (μmoles Ca^{2+}/g protein)	(‡)	1.09 ± 0.11	1.14 ± 0.13	1.03 ± 0.13	1.20 ± 0.11
Myosin ghost myofibrils (μmoles Ca^{2+}/g protein)	(‡)	2.38 ± 0.26	2.66 ± 0.33	2.18 ± 0.17	2.38 ± 0.22
Myofibrils (μmoles Ca^{2+}/g protein)	(‡)	0.75 ± 0.13	0.76 ± 0.15	0.87 ± 0.21	0.66 ± 0.12

* Values are means ± S.D. from four measurements. Other conditions were the same as those in Fig. 1B.
† Chelex resin method; free Ca^{2+} = 0.51 μmole.
‡ Equilibrium dialysis method; free Ca^{2+} = 2 μmoles.
§ Equilibrium dialysis method; free Ca^{2+} = 10 μmoles.

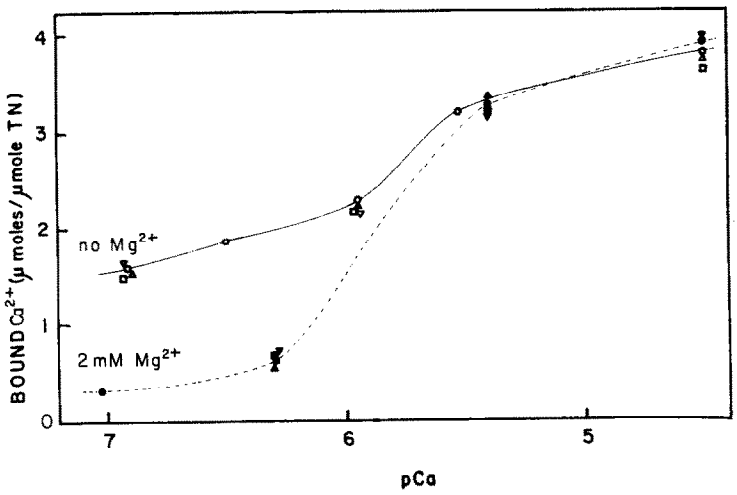

Fig. 3. Effects of ethanol and ADP on Ca^{2+} binding by troponin, measured by equilibrium dialysis. Equilibrium dialysis was carried out overnight at 25°. The concentration of troponin was 1 mg/ml. Experimental conditions were the same as shown in Fig. 1. Open symbols (solid line) represent the absence of Mg^{2+} and closed symbols (dashed line) the presence of 2 mM Mg^{2+}; control (○, ●), 0.5% ethanol (△, ▲), 100 μM ADP (▽, ▼) and 0.5% ethanol plus 100 μM ADP (□, ■).

Table 3. Ca^{2+} binding of actomyosin in the absence and presence of 2 mM ATP*

		Ca^{2+} (μmoles/g protein)			
Method	ATP	Control	Ethanol (1%)	ADP (0.1 mM)	ADP + ethanol
Millipore filtration	None	0.88 ± 0.09	0.91 ± 0.08	0.84 ± 0.08	0.92 ± 0.09
	2 mM	0.71 ± 0.08	0.39 ± 0.05	0.43 ± 0.04	0.52 ± 0.04
Centrifugation	None	0.98 ± 0.12	0.92 ± 0.11	0.94 ± 0.10	0.96 ± 0.14
	2 mM	0.78 ± 0.09	0.53 ± 0.07	0.54 ± 0.05	0.73 ± 0.10

* The concentration of free Ca^{2+} was 1.0×10^{-6} M. Other conditions were the same as those in Fig. 1B. Values are means ± S.D. from four measurements.

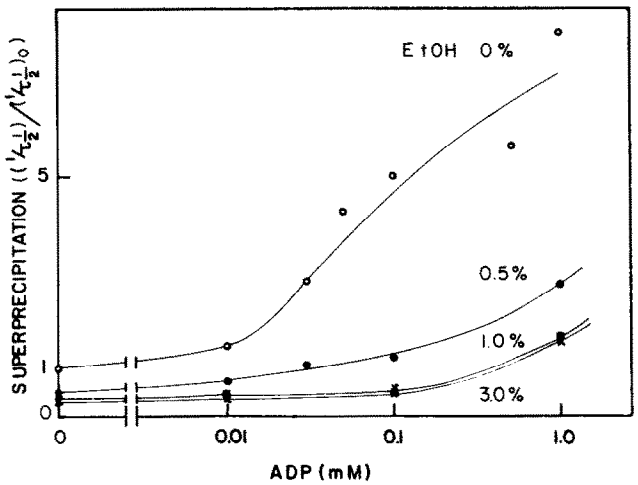

Fig. 4. Effects of ethanol and ADP on superprecipitation of an actomyosin suspension. The time needed for the optical density (at 500 nm) to reach the half-maximum value after the addition of ATP ($\tau_{1/2}$) was measured. The ratios of the experimental to control reciprocal times (control value, 3.7×10^{-3} sec^{-1}) are plotted as a function of the concentration of ethanol (0, 0.5, 1.0 and 3%) and ADP. The other experimental conditions included: 1.2 mg/ml protein, 60 mM KCl, 20 mM Tris–maleate (pH 6.8), 5 mM $MgCl_2$ and 2 mM ATP, at 25°.

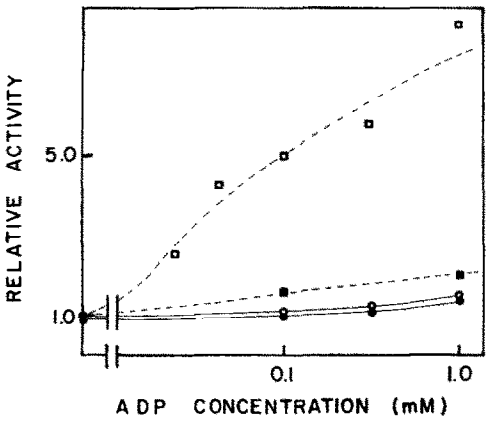

Fig. 5. Effect of the myokinase inhibitor, Ap_5A, on superprecipitation (---) and ATPase activity (——). Key: no Ap_5A (□, ○), and 0.1 mM Ap_5A (■, ●). Both activities are expressed as relative values in the figure. The ATPase activity and $1/\tau_{1/2}$ in the absence of both ADP and Ap_5A, which were 88.9 moles P_i/min/g and 3.7×10^{-3} sec^{-1}, respectively, were each assigned a relative value of 1.0. The experimental conditions were the same as shown in Fig. 4.

tractile proteins, Rubin and associates [4, 5] measured three parameters, namely (a) superprecipitation of actomyosin suspension, which represents the ATP-induced physical association of actin and myosin; (b) ATPase activity, which indicates the enzymatic activity of myosin, and (c) Ca^{2+} binding of troponin, which, via tropomyosin, regulates the interaction between actin and myosin. They have shown that Ca^{2+} binding by the troponin–tropomyosin complex, as measured by the Lytron particle method, increases in the presence of ADP; that increase is inhibited by ethanol [7]. They further demonstrated that superprecipitation of isolated actomyosin and the ATPase activity of myosin are also increased by the addition of ADP. Again, these increased activities are inhibited by ethanol [5–7]. Based upon these results, it was proposed that ethanol may exert an inotropic effect, in part, by inhibiting Ca^{2+} binding by the troponin–tropomyosin complex. Since this model presents an interesting example of structure–function relationships in the regulatory mechanism of muscle contraction, we undertook the present study to elucidate the mechanism by which ADP and ethanol influence the contractile proteins of muscle. The maximum ethanol concentration which is generally seen in the blood of chronic alcoholics is about 0.5%. The concentrations in this study needed to obtain some effects are somewhat higher, but it is difficult to relate the concentration precisely to the regulated situation in the cell. Certainly the lower concentrations *in vivo* extend for far longer periods of time than is possible *in vitro*.

As shown in Fig. 1, ethanol-induced inhibition of Ca^{2+} binding by troponin–tropomyosin-coated Lytron particles was confirmed. Particles coated only with troponin did not respond to either ADP or ethanol, while the Ca^{2+} binding of troponin–tropomyosin particles was increased by the addition of ADP. The latter Ca^{2+} binding activity was inhibited by ethanol. In the absence of ADP, ethanol did not inhibit Ca^{2+} binding by troponin–tropomyosin particles. The increase in Ca^{2+} binding by troponin–tropomyosin particles was achieved by the addition of ADP, but not by the addition of other nucleotides, such as ATP and AMP (Table 1). At this time we have no explanation for this phenomenon.

We studied Ca^{2+} binding using two methods,

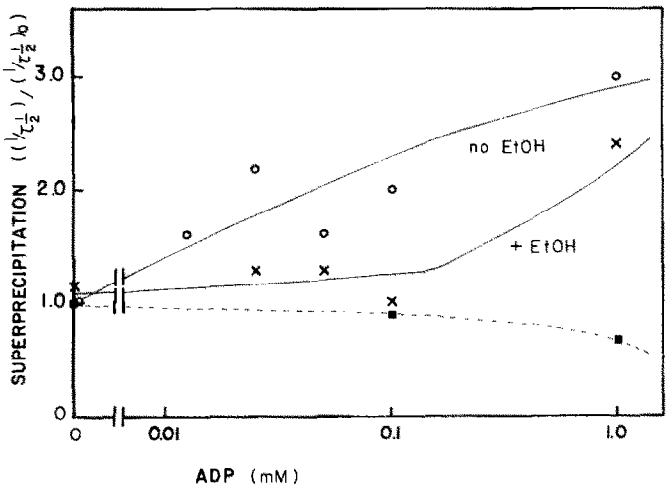

Fig. 6. Effect of a small concentration of ATP (10 μM) and various concentrations of ADP on superprecipitation in the presence of Ap_5A. ATP (10 μM) was added 5 min before the addition of 2 mM ATP in the presence (×) and absence (○) of 1.0% ethanol. The dashed line (■) denotes the absence of 10 μM ATP. Superprecipitation was determined from the changes in optical density caused by the addition of 2 mM ATP. The data are represented as the ratio of the reciprocal time ($1/\tau_{1/2}$) in the presence of 10 μM ATP to that obtained without 10 μM ATP. Control value in the absence of ADP and ethanol $(1/\tau_{1/2})_o$, was 3.8×10^{-3} sec^{-1}. The reaction mixture contained 60 mM KCl, 20 mM Tris–maleate (pH 6.8), 0.1 mM Ap_5A and 2 mM $MgCl_2$, at 25°.

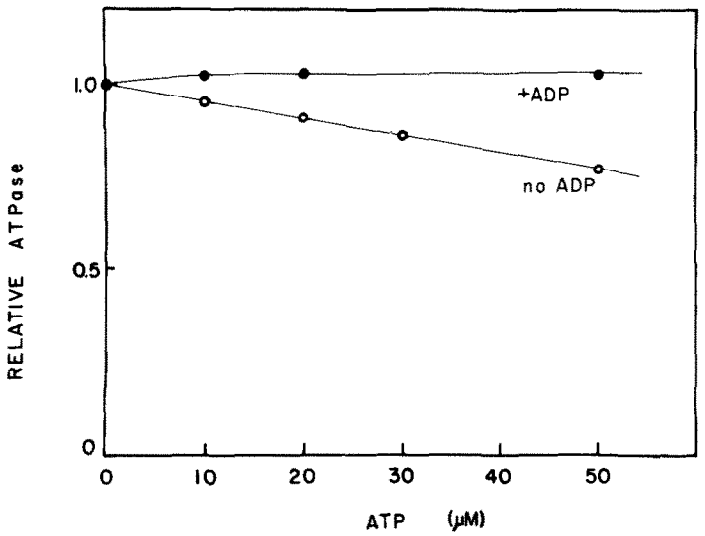

Fig. 7. Effect of a small concentration of ATP (10–50 μM) on ATPase activity in the presence of Ap_5A. ATPase activity was determined under the same conditions as in Fig. 6 as a function of a small concentration of ATP (added 5 min before the addition of 2 mM ATP). Key: no ADP (○), and 0.1 mM ADP (●). ATPase activity in the absence of ADP and a small concentration of ATP was 105 μmoles P_i/min/g, and was assigned a relative value of 1.0.

namely the chelex resin method and the equilibrium dialysis method. Ca^{2+} binding by both troponin and a troponin–tropomyosin complex in solution were not influenced by the addition of ethanol and ADP (Figs. 2 and 3, Table 3).

In the Lytron particle method, Puszkin and Rubin [7] used a troponin–tropomyosin complex adsorbed on the solid surface of Lytron particles. If the structural configuration of tropomyosin and troponin is important, we might expect to observe a similar effect in actomyosin or muscle fiber preparations. For this purpose we used three systems: (a) an actomyosin suspension, (b) myosin ghost myofibrils, and (c) myofibrils. As shown in Table 2, neither ADP nor ethanol influenced the Ca^{2+} binding of these preparations. Since these measurements were made in the absence of ATP, the myofibril preparations were in the "rigor state". We, therefore, also studied the Ca^{2+} binding of actomyosin in the presence of ATP (contracted state). As shown in Table 3, no effects of ADP and ethanol were observed with the millipore filtration and centrifugation methods in the absence of ATP. In the presence of ATP, both ADP and ethanol inhibited Ca^{2+} binding. Thus, contrary to the results obtained with the troponin–tropomyosin–Lytron particle complex, the enhancement of Ca^{2+} binding by ADP was not observed in these systems.

According to Fig. 1, the maximum level of troponin Ca^{2+} binding was approximately three Ca^{2+} per troponin molecule. Since this value reaches the theoretical value of four in other experiments (Figs. 2 and 3), it appears that the absorption of troponin onto the surface of Lytron influenced the Ca^{2+} binding properties of troponin. It is possible that the observed effects of ADP and/or ethanol on the Ca^{2+} binding of troponin is derived from the modification of protein–Lytron interaction by these agents. Although the modification of protein–Lytron interaction by ADP and ethanol may provide an interesting model to study the mechanism of conformational change of the troponin–tropomyosin system, it may not necessarily be related to the regulatory mechanism of muscle contraction.

We studied the effects of ADP and ethanol on the superprecipitation of actomyosin. As shown in Fig. 4, ADP increased the velocity of superprecipitation, while ethanol inhibited that enhancement. Under these conditions, ethanol also inhibited superprecipitation in the absence of ADP (Fig. 4). Because muscle preparations display myokinase activity [22], we studied the influence of ADP on the actomyosin system by producing ATP plus AMP via myokinase activity. The enhancement of superprecipitation caused by 0.1 mM ADP was abolished in the presence of Ap_5A (Fig. 5). In the present experimental procedure, as well as that used in other studies [5–7], ADP was added 5 min prior to the addition of ATP. It is, therefore, possible that small amounts of ATP appear in the system, thereby enhancing the association of actin and myosin *in vitro* upon the addition of ATP. A small concentration of ATP (10 μM) added 5 min before the addition of 2 mM ATP increased the velocity of superprecipitation in the presence of ADP. Superprecipitation reflects the aggregation of actin and myosin *in vitro* [23]. This aggregation is preceded by formation of centers, or nucleation. Thus, our findings suggest that a small concentration of ATP may promote such nucleation. After exposing the actomyosin suspension to 10 μM ATP for 5 min, the addition of 2 mM ATP initiated superprecipitation; the velocity of superprecipitation was increased because nuclei already existed.

The fact that ethanol inhibited the enhancement of superprecipitation caused by the addition of 10 μM ATP suggests that ethanol may inhibit this nucleation process (Fig. 6). The addition of a small amount of ATP produced a similar effect on the ATPase activity

of actomyosin, although the effect was somewhat less than that on superprecipitation (Fig. 7).

The increased velocity of superprecipitation, presumably owing to enhancement of nucleation, was only observed in the presence of ADP. Since Ap_5A prevented production of ATP in the system, ADP apparently does not act by producing ATP. It has been reported that the binding of Mg^{2+}-ADP to myosin brings about a change in the myosin cross-bridge conformation [24]. The enhancement of nucleation may be related to this conformational change. Further studies are needed to elucidate this mechanism.

In summary, the data presented in these studies raise questions regarding the adequacy of superprecipitation *in vitro* as a model for muscle contraction *in vivo*, since nucleation is not required *in vivo*.

The data also suggest that, although both ADP and ethanol may have some effects on contractile proteins, the site of actions is not related to Ca^{2+} binding of troponin.

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