Adenosine Diphosphate Requirement in the Creatine Phosphate–Induced Contraction of the Glycerinated Rabbit Psoas

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ABSTRACT Glycerinated rabbit psoas fibers were tested for their ability to contract under the influence of creatine phosphate and creatine kinase in the absence of free nucleotide. Tension development by the fibers was observed upon addition of creatine phosphate to the medium containing creatine kinase purified to the first lyophilization stage. However, when the enzyme was washed free of nucleotides by treatment with the anion exchange resin Dowex 1, no contraction occurred until free nucleotide was supplied. In all experiments, contractile activity of the psoas fibers was the criterion for determining the enzyme activity concerned. Using this criterion, creatine kinase activity native to the glycerinated fibers was also demonstrated. No evidence for direct transphosphorylation of the bound nucleotide of the fiber was found.

INTRODUCTION

The "mechanochemical coupling" in muscle contraction has come into prominence recently through the work of Carlson and Siger (1). They suggest a path of entry of phosphate bond energy into the contractile elements of muscle *via* "compartmentalized" actin. The bound nucleotide of the actin is phosphorylated in their scheme by the sarcoplasmic CP-CPT system.[1] This mechanism has received inferential support from the findings of Yagi and Noda (2), who showed that when CP and CPT were added to a suspen-

[1] The following abbreviations are used throughout the text:
ATP = adenosine triphosphate
ADP = adenosine diphosphate
CP = creatine phosphate
CPT = ATP-creatine phosphotransferase, creatine kinase
tris = tris(hydroxymethyl)aminomethane

sion of myofibrils, a release of free creatine resulted. The results were obtained with myofibrils pretreated with deoxycholate, and with myofibrils prepared from glycerinated muscle. In the latter case, these authors made the interesting observation that under conditions of free creatine release, a slow contraction of the fibrils occurred.

It is of significant interest that in the foregoing observation, the contraction occurred without the participation of nucleoside triphosphates added to the system. Such a contraction with an intact muscle fiber instead of myofibrils, if demonstrable, would not only provide confirmation for Yagi and Noda but also make available a system in which the contraction itself could be measured quantitatively as an approach to the factors involved in mechanochemical coupling. For this reason we undertook this investigation.

MATERIALS AND METHODS

Preparation of Glycerinated Rabbit Psoas Fibers

Thin bundles of psoas fibers were glycerinated as described by Yagi and Noda (2), using cotton-tipped glass rods. These fiber bundles were kept in a 50 per cent (v/v) glycerine solution and stored in a deep-freeze at −23°C without further treatment for 2 to 10 months before use. For the experiments, a bundle cut loose from the supporting rod was teased apart in a Petri dish containing 20 per cent (v/v) glycerine. With watch-maker's forceps it was possible to obtain a two fiber bundle, with which diffusion effects were minimized. The double stranded structure and fiber integrity were checked microscopically before use. These double fibers will be referred to as "fibers" in the succeeding text.

Preparation of ATP-Creatine Phosphotransferase (CPT)

CPT was prepared and its activity assayed according to the method of Noda, Kuby, and Lardy (3). The dialyzed extract of rabbit back and leg muscles was lyophilized but not crystallized. The product had an activity of approximately 30 units per milligram of preparation. The lyophilized extract was stored in a deep-freeze at −23°C until use.

For specific experiments, portions of the enzyme preparation were treated with Dowex 1, an anion exchange resin (polystyrene with —NMe_3^+ groups), which may be used to remove contaminating nucleotides (4, 5). CPT was dissolved in water, the pH adjusted to 9.0 with KOH, and a portion of the solution passed through a column of Dowex 1 X2 resin (50 to 100 mesh, in the chloride form; column 1 cm diameter and 3 cm high and maintained at 0–5°C). This was followed by a wash of the column with water. The resin-treated CPT solution and the water wash were collected together to a measured volume and stored at 0°C for up to 7 days during use, along with the remaining portion of the untreated CPT solution. Nitrogen determinations by a modification of the microKjeldahl method were made on samples of resin-treated and untreated CPT solution to ascertain the degree of recovery of protein from the column.

Two batches of CPT were resin-treated, one being used in experiments in tris medium, the other in borate histidine medium. Details in the preparation are as follows. For the tris batch, 10 ml of CPT solution at 2.5 mg/ml (note: CPT weight values given here and throughout this paper are not corrected for enzyme purity) were passed through the column and followed by a 6 ml water wash. Nitrogen recovery was 87 per cent. For the borate-histidine batch, 7 ml of CPT solution at 12 mg/ml were passed through the column followed by a 4.5 ml water wash, with a

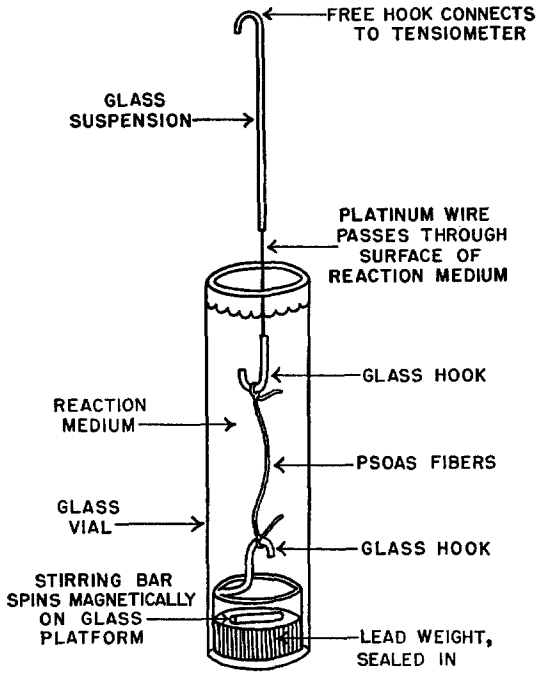

FIGURE 1. Diagram of reaction vessel and contents.

10.5 ml total volume collected. Nitrogen recovery was 90 per cent. For both batches, 1 ml water was retained by the column.

Apparatus and Procedure for Measuring Isometric Tension Development

A quartz lever tensiometer described by Hayashi *et al.* (6) was employed with these modifications: a glass vial (6.4 cm high × 1.8 cm outside diameter) served as the reaction vessel; temperature was controlled by a closed circulating 30°C water bath surrounding the reaction vessel; continuous stirring was achieved magnetically (Fig. 1).

One end of the fibers was tied with a double knot onto a weighted glass hook and the other end similarly to a glass suspension hook (refer to Fig. 1). The hooks were then linked together and the weight picked up by forceps and gently dropped directly into the vessel containing the incomplete reaction mixture (generally lacking CP), During the next 15 to 20 minutes the free hook was connected to the tensiometer,

the fiber hooks disengaged, a stirring bar added, and the fibers adjusted to zero per cent slack and zero tension. (Minor background fluctuations in tension readings, sometimes producing negative tensions, result from the sensitivity of the quartz lever to slight shifts in the alignment of fibers and suspension hooks which may be caused by air currents, magnetic stirring, occasional formation of a small gas bubble on hook or fiber, or by the addition of a volume to the vessel already containing a suspended fiber.) Length of the fibers from hook to hook varied in different experiments from 0.50 cm to 1.38 cm as measured by a telescope vernier arrangement. Tension measurements in the incomplete reaction mixture were recorded for 15 minutes, with the first reading presented as a tension of zero milligrams at time zero on the graphs below. This equilibration period, showing background tension fluctuations, was followed by the addition of the mixture-completing component (generally CP) and continued tension measurements.

Composition of Contraction Media

Experiments were conducted in two different media. Tris medium was 0.025 M tris, containing 0.05 M KCl and 0.001 M $MgCl_2$ adjusted to pH 6.8 with HCl. Five mg of untreated or resin-treated CPT was usually included. For individual incubations, 0.5 ml of 10 mM CP was added to 8.5 ml of tris medium, completing the reaction mixture and bringing the final concentrations to those stated. Final CP concentration was 0.556 mM.

Borate histidine medium consisted of a 0.03 M histidine, 0.018 M borate mixture (pH 7.1) containing 0.0135 M KCl, 0.001 M $MgCl_2$, and 0.05 mM glycine. Depending on the particular experiment, 5 mg or 23.5 mg of CPT was included. For individual incubations, addition of 0.5 ml of 10 mM CP to 7.5 ml incomplete borate-histidine medium completed the mixture, bringing the final concentrations to those given, and final CP concentration to 0.625 mM. This medium with 23.5 mg CPT approximates the one used by Yagi and Noda (2) in their experiments on the creatine-liberating activity of myofibrils isolated from glycerinated rabbit psoas fibers, except that they used crystalline CPT.

Many experiments required various additions to these media; details are presented with the graphs showing specific experiments and results.

The water used throughout the work was laboratory-distilled water deionized by passing through a column of Amberlite MB-3 ion exchange resin. CP was obtained from the California Corporation for Biochemical Research; ADP and ATP as the sodium salts, from the Sigma Chemical Company.

EXPERIMENTS AND RESULTS

Incubations in Tris Medium

In the first experiments, employing untreated CPT, the application of the CP-CPT system to fibers glycerinated for 5½ months evoked a contraction as a response (Fig. 2, curve 1. Note that in all graphs, an addition to the medium is indicated by a notation within a box; for example, in Fig. 2, ●○CP in box denotes addition of CP to media represented by curves 1

and 2.) However, if the fibers were incubated with CPT which had been treated with Dowex 1, the contraction with CP was eliminated (Fig. 2, curve 2). To determine whether the presence of free nucleotide could restore contraction, ADP was added subsequently to these fibers; 0.01 μmole of ADP had no effect, but tension developed in response to further addition of 0.1 μmole of ADP.

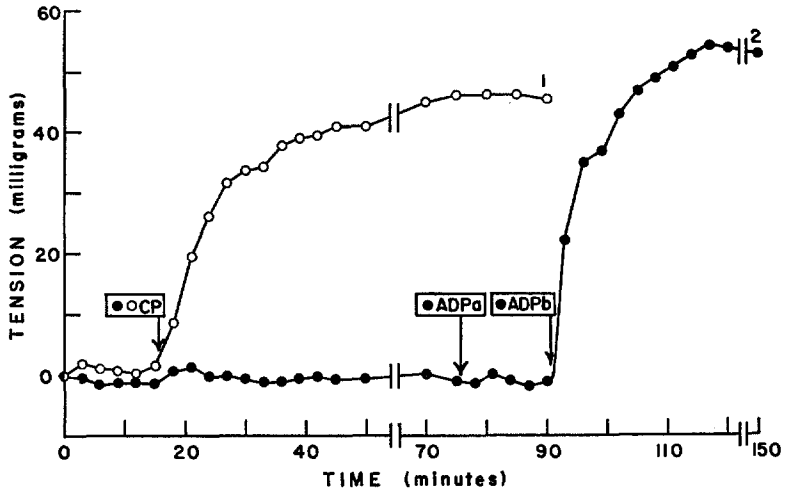

FIGURE 2. CP and ADP requirements for isometric tension development in fibers incubated in tris medium containing 5 mg untreated CPT compared with 5 mg CPT treated with Dowex 1 resin. Fibers glycerinated 5½ months. The following additions were made at the times indicated on the graph: CP, 5 μmoles in 0.5 ml H_2O; ADPa, 0.01 μmole in 0.1 ml tris medium; ADPb, 0.1 μmole in 0.1 ml tris medium. Curve 1, untreated CPT. Curve 2, resin-treated CPT. Similar results were obtained in a repeat experiment of each type.

Control experiments to show that the CPT had an active role in the above contractions were done by omitting the CPT from the incubation medium. The addition of CP and ADP now had little effect. A trace of resin-treated CPT added to the medium at the end of one experiment produced a slow contraction, demonstrating the activity of the resin-treated enzyme (Fig. 3, curve 1). ATP added at the end of a second experiment produced a sharp contraction (Fig. 3, curve 2), showing that the fibers had retained substantial capacity for tension development.

Incubations in Borate Histidine Medium

A second set of similar experiments was done in borate histidine medium since this was the medium in which the CP-CPT system had produced contraction in myofibrils isolated from glycerinated muscle (2) and also since it seemed desirable to look for any possible buffer effects.

Five mg of untreated CPT was used in each of four preliminary experiments (fibers glycerinated for 2 months) in which the time of addition of CP or CPT was varied. In all four, contraction occurred when and only when both CPT and CP were present; added nucleotide was not required. Values for maximum tension varied from 35.2 to 44.8 mg. In two controls,

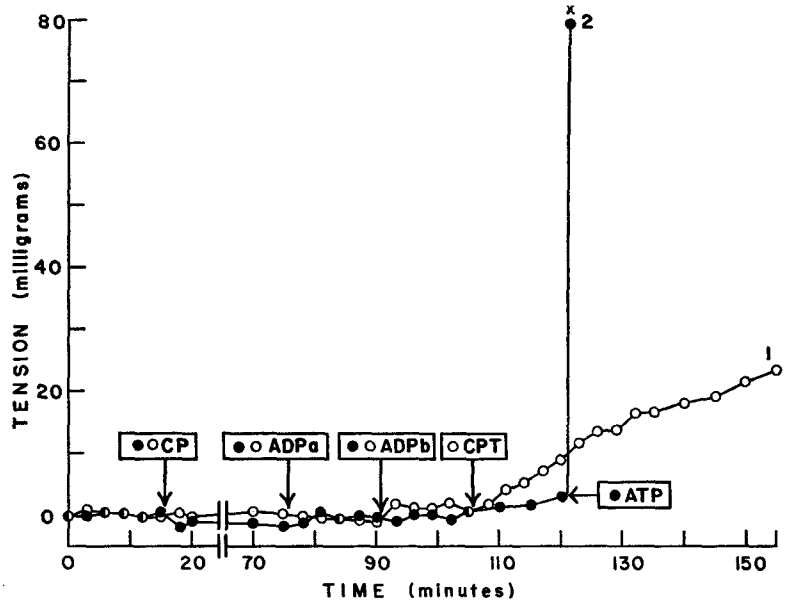

FIGURE 3. Effect of omission of CPT from tris medium. Fibers glycerinated 5½ months. Additions of CP and ADP as in Fig. 2. Curve 1, tension development initiated by final addition of one-third mg resin-treated CPT in 0.2 ml H_2O. Curve 2, tension development produced by final addition of 20 μmoles ATP in 0.2 ml H_2O. X indicates point at which fibers break.

one containing no CPT and the other containing heat-denatured CPT, contraction was not obtained.

When fibers glycerinated for 4 months were incubated with 23.5 mg untreated CPT, the same result was obtained (Fig. 4, curve 1). However, as in tris medium, incubation with resin-treated CPT eliminated the contraction produced by CP addition; again, tension development was restored by addition of ADP (Fig. 4, curve 2). In a control experiment, the CPT was omitted. The addition of CP had little effect, but the subsequent addition of ADP produced a strong contraction (Fig. 4, curve 3). Thus, these fibers seemed to contain a native enzyme which was ADP-dependent.

With fibers glycerinated 7 months, resin treatment of the CPT again eliminated CP-induced contraction, and made it dependent upon ADP addition. In the control experiment, native enzyme activity provided a strong contraction which was somewhat slower than that shown in Fig. 4. In another

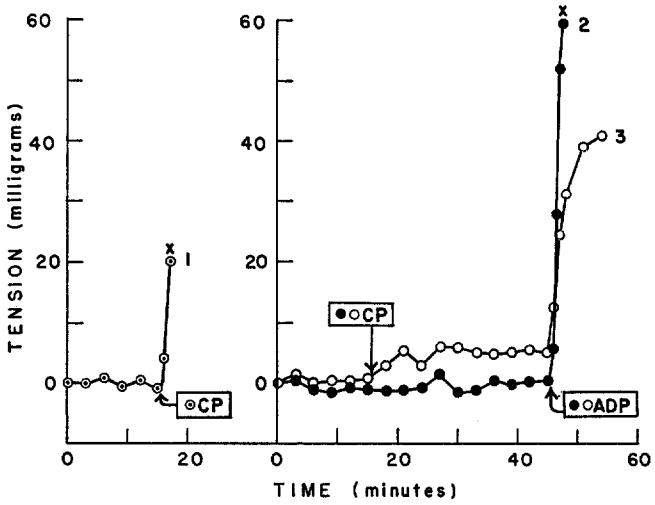

FIGURE 4. CP and ADP requirements for isometric tension development in fibers incubated in borate-histidine medium containing 23.5 mg untreated CPT, 23.5 mg resin-treated CPT, or no CPT. Fibers glycerinated 4 months. Additions: CP, 5 μmoles in 0.5 ml H_2O; ADP, 0.1 μmole in 0.1 ml H_2O. Curve 1, untreated CPT. Curve 2, resin-treated CPT. Curve 3, no CPT. X indicates point at which fibers break.

control experiment, the order of substrate addition was reversed. The first addition of ADP had no effect, but the subsequent addition of CP produced a contraction, showing the native enzyme to be dependent on both CP and ADP, but not on either alone (Fig. 5, curve 1). Thus it conformed to the substrate requirements of CPT. Although not included in the figures, similar experiments showed this CP- and ADP-dependent native activity to be

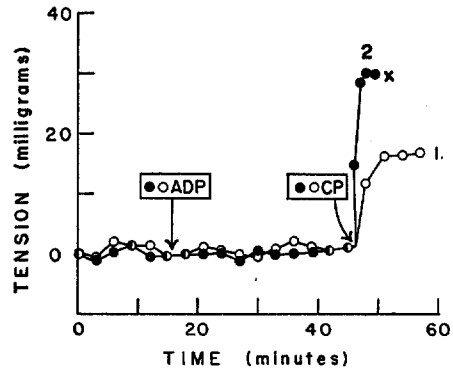

FIGURE 5. Dependence of isometric tension development upon addition of CP when fibers are incubated in borate-histidine medium containing 23.5 mg resin-treated CPT, or no CPT. Fibers glycerinated 7 months. Additions of CP and ADP as in Fig. 4, but in reverse order. Curve 1, no CPT. Curve 2, 23.5 mg resin-treated CPT. X indicates point at which fibers break.

present in fibers glycerinated for 10 months (four experiments were done, two in which ADP was added before CP, and two with CP added before ADP).

In a final control experiment, the resin-treated CPT was tested for myokinase activity. The fibers were incubated first with resin-treated CPT, and addition of ADP produced no contraction. Subsequent addition of CP, however, produced a strong contraction (Fig. 5, curve 2).

It is important to note that in each of the experiments graphed, contraction of the fibers was obtained by some means before the experiment was concluded. Thus, all fibers were potentially contractile throughout incubation.

DISCUSSION AND CONCLUSIONS

Early experiments on the effect of phosphagens on isolated fibers did not produce information bearing on the problem at hand, principally because of uncontrolled factors and because the problem had not been specified for the experimenters (7–9). The problem can be presented as follows: can the bound nucleotide of the myofibril be directly phosphorylated by a transphosphorylating system as a part of the mechanism of contraction? In experiments using myofibrillar suspensions of fresh muscle, Perry (10) found that CP and CPT did not induce either contraction or creatine release; however, if he added small amounts of ADP a rapid contraction of the myofibrils ensued. An equivalent amount of ATP alone had no effect. He concluded that the bound nucleotide of the fibril was not available to direct phosphorylation by the CP-CPT system. Yagi and Noda (2) obtained creatine release from myofibrils pretreated with deoxycholate, and also from fibrils obtained from glycerinated muscle. They concluded that the bound nucleotide was directly phosphorylated by the CP-CPT system.

The results presented herein show that intact fibers do not respond to CP-CPT if the enzyme preparation is cleaned of contaminating nucleotides through the use of Dowex 1. Thus, our results agree with those of Perry (10).

Actually, Bozler (11), using bundles of glycerinated fibers, obtained results similar to those of the present experiments, including the demonstration of native CPT activity. However, his experiments were not designed toward answering the same questions, and therefore are inapplicable to these questions.

The possibility exists that the results of Yagi and Noda can be explained by assuming that either ATP or ADP was present as a contaminant in their CPT preparation. However, this seems unlikely since they obtained no effect with fresh myofibrils in the presence of CP and CPT. The problem then becomes one of explaining the difference in results using intact glycerinated fibers as compared with the results of Yagi and Noda using glycerinated myofibrils.

It might be argued that with the intact fibers, added CPT is unable to penetrate to the site of the bound nucleotide. However, even if this were true, the finding that in borate histidine medium the fibers already contain enough active CPT to cause a contraction and that such a contraction is still dependent upon addition of ADP (Figs. 4 and 5; curves 3 and 1, respectively) necessitates forwarding the additional argument that sufficient native CPT is not available to the bound nucleotide. The absence of this native activity in fibers in the tris medium has no explanation as yet. It may be a specific buffer effect, or a possible washing out of the native enzyme during the longer incubation periods employed in the tris experiments.

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