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KINETIC PROPERTIES AND FUNCTIONAL ROLE OF CREATINE PHOSPHOKINASE IN GLYCERINATED MUSCLE FIBERS - FURTHER EVIDENCE FOR COMPARTMENTATION

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The following phenomena were observed when relative contraction and relaxation effects of ATP and creatine phosphate (CP) were studied in rabbit psoas muscle glycerinated fiber bundles containing native creatine phosphokinase (CPK) and ATPase activities: (1) nucleotide was absolutely necessary for contraction; (2) in the presence of a small amount of ADP (250 μM), physiological concentration of CP (10 mM) produced faster and stronger contraction and faster, more complete, relaxation than equimolar or higher concentrations of ATP; (3) if the nucleotide was in the form of ATP, the nucleotide K_m for contraction was about 1.5 mM; (4) if the nucleotide was in the form of ADP, the nucleotide K_m for contraction at physiological concentration of CP (10 mM) was 0.076 to 1.18 mM depending upon the order of addition of ADP and CP; (5) the apparent K_m for CP for contraction was 2.67 mM independent of sequence of addition of ADP and CP.

The localization of CPK isoenzymes in mitochondria and myofibrils and their functional coupling with adenine nucleotide turnover are the important features of mitochondrial myofibrillar shuttle of creatine phosphate and creatine (1-5). The existence of this shuttle has been further supported by the kinetics, biochemical (6-8) and labeling studies (9).

All previous quantitative work on this problem however has been done using chemical assays of ATP or CPK activity or measurement of intermediates. The true functional measure of the role of compartmentation must be by its role in muscle contraction. Since all the experiments reported on contraction of glycerinated fibers have been done in the presence of added CPK (10-12), we wish to report here that there is adequate endogenous CPK activity in glycerinated fibers to support normal contraction and relaxation with CP and small amounts of ADP. We also report here data on compartmentation of CPK and ATPase in glycerinated fibers as determined by comparison of K_ms for ATP, ADP and CP for rate of tension development in glycerinated muscle

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fibers with those for CPK in homogenized fibers. The present work provides further evidence for the compartmentation of myofibrillar CPK and ATPase as part of the creatine-creatine phosphate energy shuttle (2).

MATERIALS AND METHODS

Glycerinated rabbit psoas muscle fiber bundles were prepared according to the method of Hanson and Huxley (13) with slight modification, in which 10 mM maleate-K buffer, pH 7.0, replaced 6.7 mM phosphate buffer. For each experiment fiber bundles were teased into small bundles of 0.3-0.4 mm diameter. Bundles of uniform diameter throughout their length were selected with an optical comparator. Each bundle was cut into two equal lengths of about 1.8 cm. Preliminary experiments showed that the responses of pairs of bundles prepared this way were within 5% in rate and force of contraction to the same stimulus. The apparatus used consisted of a 3 ml plastic disposable syringe barrel with the proximal flange removed. A removable wire rack with a bottom and top hook was designed to fit tightly in this barrel. At the bottom was a small Teflon covered magnetic stirring bar. After a bundle of fibers was tied to the bottom hook of the rack and set in the barrel it was washed three times with stock contracting solution containing 100 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris, pH 7.0. Then 1.72 ml of this solution was placed in the bath. The fibers were put under 150 mg tension to keep them straight. Whenever the inhibitor of myokinase, P^1,P^5 adenosine-5'-pentaphosphate (AP5A) (14) was used, the fibers were preincubated with it for one minute. 25 μM AP5A was enough to inhibit the contraction of more than 2 month old glycerinated fibers in the presence of more than 5 mM ADP, whereas higher concentrations, up to 200 μM AP5A were necessary for complete inhibition of fresher fibers. The contraction was initiated by addition of CP, ADP or ATP and allowed to reach maximum height. Then 3 mM EGTA for 10 μM $CaCl_2$ and 33 mM EGTA for 100 μM $CaCl_2$ was added for relaxation. Preliminary experiments showed that 10 μM $CaCl_2$ was enough for contraction of more than 2 month old glycerinated fibers but higher concentrations were necessary for fresher fibers. The rate of contraction was measured as $gm/s/cm^2$ from the linear portion of the contraction curve. All the experiments were carried out at room temperature (20-21°C).

The CPK activity was determined in the direction of ATP formation by measuring creatine liberation. The reaction was carried out by addition of 100 μl of fiber homogenate (about 10 mg protein/ml) to a mixture of 0.76 ml of stock contracting solution, 10 mM CP, 10 μM $CaCl_2$ and 5 mM ADP to final volume of one ml and incubated at 29°C. The reaction was stopped by addition of 0.5 ml of 1.4 M $HClO_4$. Protein was determined by the Lowry method (15). Creatine formed was measured (16) in the supernatant solution after it was neutralized by the Freon/Alamine method of Khym (17).

RESULTS

The activity of CPK in glycerinated fibers diminishes rapidly in the first two to three weeks and plateaus after three weeks, staying at the level of 1.8±2 IU (IU = μmoles/min/mg protein). These fibers were used to determine the apparent K_m for nucleotides and CP for the rate of contraction. In one set of experiments, contraction was initiated by addition of 20 μl of ADP solution into the bath containing 10 mM CP and 10 μM $CaCl_2$. Half the maximum tension development was attained at the concentration of 1.18±0.24 mM ADP.

TABLE I: Apparent K_ms for CPK on the Various Conditions

Conditions	K_ms (mM) ADP	CP
Contraction initiated by addition of ADP in presence of CP	1.18±.24	2.76±0.6
Contraction initiated by addition of CP in presence of ADP	0.077±.004	2.63±0.58
Soluble CPK	0.151±.012	2.68±.02

When the fibers were preincubated with ADP in the presence of 25 μM AP5A and contraction initiated by addition of 10 mM CP, the K_m for ADP was 76.9±4 μM. In this case only 250 μM ADP was enough to give maximum rate of contraction in these fibers (812.8±73.6 gm/s/cm^2) in contrast to 10 to 12 mM in the previous case. This K_m value is about 15 times smaller than the value obtained when contraction was initiated with ADP, and is about half the value for CPK activity in homogenized glycerinated fibers (151.5±12 μM). The K_m for CP for the initial rate of tension development was the same (2.67±0.05 mM), whether the fibers were preincubated with CP and contraction initiated by addition of ADP or vice versa. The same K_m for CP was also obtained when CPK activity was measured in homogenized glycerinated fibers. When contraction was initiated by addition of ATP alone, the K_m of about 1.5 mM was obtained for ATP for rate of tension development. The data are summarized in Table I.

When the contraction responses of these glycerinated fibers to ATP and CP +ADP were compared, it was found that in the presence of 10 mM CP, ADP concentrations as low as 4 μM were able to produce contraction. In contrast 100 μM ATP alone was necessary in order to initiate even slight contraction (Fig. 1).

Since 250 μM ADP in the presence of 10 mM CP was able to produce maximum contraction response, the bundle of fibers was preincubated with 250 μM ADP in the presence of AP5A and contraction and relaxation responses of these fibers to equal amounts of "phosphate energy" provided by either CP or ATP were compared. In this experiment (Fig. 2) contraction was initiated by addition of either ATP, CP or ATP+CP. The slopes in this figure show the rate of

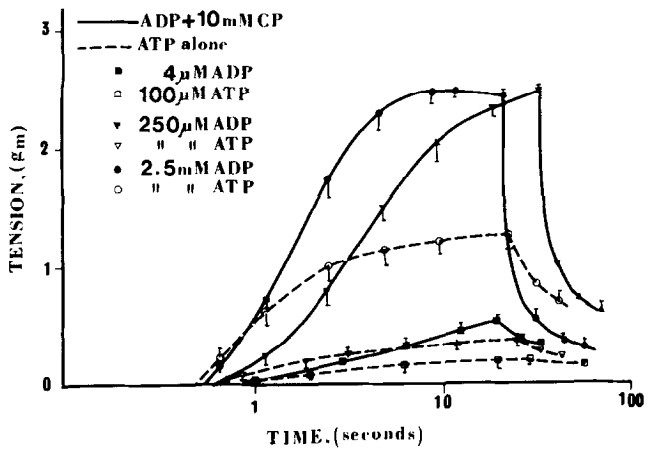

Fig 1: Comparison of isometric contraction and relaxation of glycerinated fiber bundles with ADP+CP and ATP alone plotted on semilog paper.

tension development and relaxation. The rate of tension development and relaxation were both much higher when 10 mM CP was present.

DISCUSSION

In 1956 Perry (18) showed that in the presence of added CPK and excess CP, ADP was able to cause the same qualitative shortening of myofibrils as ATP at 2 to 3 higher orders of concentration. The experimental design, using CP to initiate contraction in myofibrils with added CPK, did not permit a compari-

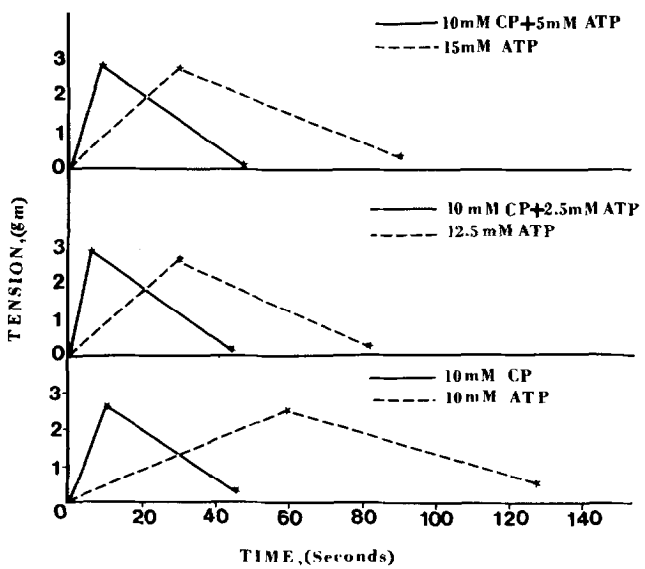

Fig. 2: Contraction and relaxation rates of glycerinated fibers with equimolar ATP or CP. All fibers were preincubated with 250 μM ADP and contraction initiated by addition of either ATP, ATP+CP or CP.

son of the relative effectiveness of CP and ATP. This was studied in the present work by comparing the contraction and relaxation reponses of glycerinated muscle fibers caused by equal amounts of phosphoryl energy provided either by added ATP alone or CP using the fibers' native CPK. In the presence of a small amount of ADP, 10 mM CP produced much faster and stronger contraction and faster and more complete relaxation than 10 mM ATP. This provides evidence that the CP and ADP system, the fibrillar compartment of the creatine phosphate shuttle, is more efficient than ATPase alone.

The close functional interaction between CPK and myosin ATPase might be due either to physical association between these two enzymes or due to diffusional barriers for their metabolites (19). The results of the present study on the apparent K_m for ADP measured as a function of rate of tension development gave about 15 fold lower K_m for ADP when fibers were preincubated with ADP than when ADP was added to initiate contraction. Moreover this K_m for ADP was about half of that obtained for ADP for enzyme activity in homogenized glycerinated fibers. In contrast there were no significant differences in K_ms for CP in various conditions. This finding can be interpreted to mean that there is diffusional limitation for adenine nucleotides in these fibers but not for CP due to its larger size and charge/molecular ratio (20).

Data we have presented seems to deny the possibility that the differential rates of diffusion of CP and ATP are the simple explanation for their differential effects on contraction. In the case in which 4 μM ADP added in the presence of 10 mM CP caused greater contraction than 100 μM ATP, there is no question that whatever the differential effect of CP it must take place at or closely adjacent to the active site, for the concentration at the active site of nucleotide in the presence of CP could at most have been 4 μM. This concentration of ATP formed from CP was more effective than 25 times that concentration of ATP added directly. This is further supported by the data showing that the K_m for ADP for CPK in homogenized fibers is twice the K_m for contraction rate. It is apparent that the function of both CPK and myosin ATPase is different in the bound (compartmented) form than in solution.

The constant CPK activity of glycerinated fibers (26% of total CPK) and the close functional reaction between ATPase and CPK which we have shown, particularly with respect to the relative effects of CP and ATP would suggest a closer spacial relation than having the active CPK at the M-line and the ATPase distributed generally over the A-band (21).

The present findings provide evidence that the observed compartmentation of myofibrillar CPK and ATPase (7-9) seems to be due to the presence of CPK inside the myofibril near to the ATPase site, in which the rephosphorylation of contraction-produced ADP to ATP for the process of contraction and relaxation takes place much faster and more efficiently than the displacement of ADP by cytosolic ATP. All these findings support the notion of the creatine phosphate energy shuttle (2).

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