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INTIMATE COUPLING OF CREATINE PHOSPHOKINASE AND MYOFIBRILLAR ADENOSINETRIPHOSPHATASE

Samuel P. Bessman, William C.T. Yang,
Paul J. Geiger and Susan Erickson-Viitanen
Department of Pharmacology and Nutrition
University of Southern California School of Medicine
Los Angeles, CA 90033

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Summary: ATPase and creatine phosphokinase (CPK) activities of isolated cardiac myofibrils were determined with ^{32}P γ-labeled ATP alone and with the addition of phosphorylcreatine (PC). With ATP and PC as substrates the label in the inorganic phosphate formed is greatly diluted indicating that the ATP formed by PC through CPK can reach the ATPase active site more readily than labeled ATP from the medium. The tight coupling of the ATPase and CPK activities further strengthens our view that PC serves an important role as high energy carrier between the energy producing sites (mitochondria) and the energy utilizing sites (myofibrils).

Introduction:

Respiratory control in striated muscle has been proposed to occur through a shuttle mechanism with mitochondrial phosphorylation of creatine liberated by muscle contraction (1,2). This phosphorylation is catalyzed by an isozyme of creatine phosphokinase bound to the mitochondria (3) in a configuration which permits preferred access of creatine to mitochondrially generated ATP. Evidence has been obtained for such preferred access (4) and the conclusions are supported by kinetic analyses (5).

Turner (6) demonstrated the presence of an isozyme of CPK (MM) on the myofibril in the region of myosin and Sharov et al as well as Mani et al have shown that this isozyme is distributed throughout the A band (7,8). Saks et al (9) found that purified myofibrils contained both CPK and ATPase activities and the CPK activity increased with increasing concentrations of ATP. The close interaction between ATPase and CPK was predicted on the basis of the kinetically favored formation of ATP from PC, and the localization of these two

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Abbreviations: CPK, creatine phosphokinase (EC 2.7.3.2); PC, phosphorylcreatine; SA, specific activities; cpm, counts per minute.

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enzymes in the myofibrils. We report herein that there is a preferential liberation of P_i by ATPase from ATP generated from PC as catalyzed by CPK. Thus, in the presence of PC,ADP is rapidly converted to ATP by myofibrillar CPK. The newly formed ATP quickly gains access to the myofibrillar ATPase site resulting in the release of P_i. Our experimental evidence therefore provides direct support of the role of PC in energy transport at the energy utilization sites in cardiac fibers.

Methods:

Materials: All reagents were of highest purity obtainable. Phosphorylcreatine was purchased from Sigma Chemical Co. New England Nuclear or ICN (Irvine, CA) supplied the ATP [γ-^{32}P]. Alamine-336 is a product of General Mills Chemicals, Inc. Freon-TF was obtained from Miller-Stephenson Chemical Co., Inc.

Reaction System: Isolated rabbit heart myofibrils were prepared according to the method of Solaro et al (10). Incubations were carried out in 1 ml reaction volumes of the following composition: Imidazole buffer 30 mM,pH 7.0; $MgCl_2$, 2 mM; ATP, 1.6 mM containing 15 µCi of ^{32}P-labeled ATP; and PC, 1.6 mM when appropriate. Myofibrils equivalent to 2-3 mg of protein were used to start each individual reaction after each reaction vessel had been preincubated in the Dubinoff shaker at 28° C for 5 minutes. The reactions were terminated immediately and after 1 and 3 min. by the addition of 1.0 ml of 1.4 M perchloric acid. Supernatant solutions containing the acid soluble phosphorylated compounds were decanted from the centrifuged protein pellet and extracted with 2/1:v/v Freon-Alamine-336 (11) which removes perchloric acid. The pH value of the extract was adjusted to neutrality to bromothymol blue with a small amount of potassium bicarbonate. The myofibrillar protein content was determined by the method of Lowry et al (12).

Chromatography of extracts and ^{32}P counting: Separation of PC, ADP, ATP and P_i and their determination were carried out with the use of the Bessman phosphate analyzer, as previously described (4). Counting was accomplished in a Delta-300 (Searle Analytic) Scintillation counter with the use of Scintiverse (Fisher Scientific, Inc.) or Phase-II (Westchem, Inc.). The phosphate analyzer is available from Alsab Scientific Products, Inc., Los Angeles, CA.

Results and Discussion

Five experiments were carried out over various time periods with essentially similar results. The results of a representative experiment are summarized in Table I. Changes in the levels of ATP, PC, and P_i confirm the presence of both ATPase and CPK activities in these myofibrils. The higher concentration of P_i at zero time with PC as additional substrate is due to P_i contamination of the commercial PC used. The rate of P_i production in the coupled reaction system (ATP plus PC as substrates) is slightly greater than

TABLE I

P_i generation by myofibrillar ATPase as effected by creatine phosphate

Substrates	Incubation Time	PC	P_i			ATP		
		nmol	nmol	cpm/nmol	total cpm	nmol	cpm/nmol	total counts
ATP 1.6 mM	0	0	1.6	7342	12408	80.3	11232	902431
	1	0	24.2	10471	248335	62.3	11203	697953
	3	0	45.2	9596	430138	44.0	10229	450206
ATP 1.6 mM	0	68.6	10.4	1482	15414	79.7	10817	862132
PC 1.6 mM	1	63.0	30.3	7847	171811	69.3	10430	722939
	3	40.8	59.1	7645	387761	61.5	7711	494922

Values represent 50 µl of the original 1.0 ml reaction medium.

that in the one substrate system at 3 minutes, which is expected as the ATP level in the latter has fallen nearly 50%. When ATP is the only substrate the specific activity (SA) of ATP is constant throughout the course of the experiment, and ATPase activity gives rise to product P_i with a SA equal to that of the γ-phosphate of ATP, as expected. Note that cpm found in P_i at time zero represent ^{32}P-labeled P_i contamination of purchased labeled ATP.

In the case of ATP plus PC as substrates for a coupled reaction system, two sources of ATP must be considered: that present at time zero in the reaction mixture, and that synthesized de novo from PC and ADP as catalyzed by myofibrillar CPK, which leads to a gradual dilution of the SA of ATP. If there is no interaction between ATPase and CPK, e.g. if the two sources of ATP mix completely, the SA of P_i at a given interval of time will approximate that of the average SA of ATP present during that reaction interval. A significant deviation from this predicted relationship implies incomplete equilibration, i.e. compartmentation of the two pools of ATP. In the experiment shown in Table I, a SA of P_i of 7847 (cpm/nmole) is to be compared with an average SA of ATP of 10623. The SA of P_i is significantly lower than that of ATP, indicating that unlabeled ATP formed through the CPK catalyzed reaction can serve directly as substrate for ATPase prior to mixing with the preexisting ATP pool. This finding is highly significant when the levels of ATP and PC present

are compared with reported kinetic constants for myofibrillar ATPase, (K_m^{ATP} = 0.16 mM); and myofibrillar CPK, (K_m^{PC} = 1.67 mM); V_{ATPase}/V_{CPK} = 2.0 (13). Thus, under conditions where the rate of CPK catalyzed ATP synthesis is relatively slow, and preexisting ATP levels are near saturation with respect to ATPase, the newly formed ATP has a direct access to the active site of ATPase. An estimate of the percentage contributions from the two ATP pools (preexisting labeled ATP versus unlabeled ATP derived from PC) may be calculated as follows for the one minute reaction interval. At the end of the interval, the amount of P_i in nmoles formed from labeled ATP can be obtained by dividing the net increase in cpm in P_i by the SA in cpm/nmole of ATP at zero time.

$$\% \text{ Labeled } P_i = \frac{\dfrac{\text{Net increase in cpm in } P_i \text{ at 1'}}{\text{cpm/nmole of ATP at zero time}}}{\text{Net } P_i \text{ released in nmoles at 1'}} \times 100$$

$$\% \text{ Unlabeled } P_i = 100 - \% \text{ Labeled } P_i$$

The labeling pattern of P_i may then be compared to the respective percentages of labeled and unlabeled ATP which can be calculated similarly.

$$\% \text{ Labeled ATP} = \frac{\dfrac{\text{cpm in ATP at 1'}}{\text{cpm/nmole of ATP at zero time}}}{\text{nmole ATP at 1'}} \times 100$$

$$\% \text{ Unlabeled ATP} = 100 - \% \text{ Labeled ATP}$$

The results of 6 experiments calculated in this manner for the one minute reaction interval are shown in Table II. For this interval, labeled ATP con-

TABLE II

Comparative P_i production and composition of ATP pool from added ATP and ATP generated from PC

	Formed from labeled ATP in %	Formed from Phosphorylcreatine %
P_i present after 1 min.	70.6±3.8 (6)*	29.4±3.7 (6)
ATP present after 1 min.	93.1±0.9 (6)	6.9±0.8 (6)

* Each value represents the mean ± S.E.M.
Number in parentheses indicates the number of experiments.

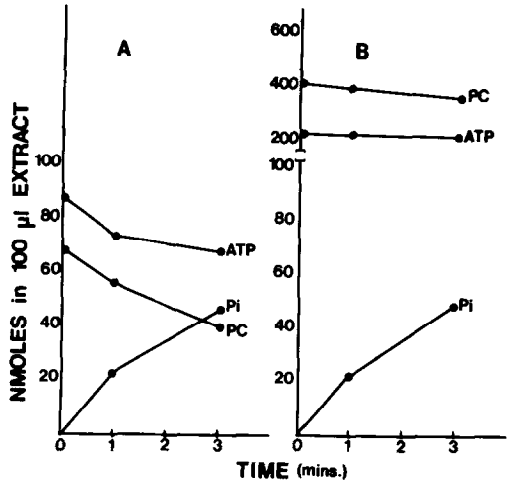

Fig. 1: Substrate concentrations of ATP and CP on coupled activities of myofibrillar ATPase and CPK.
A. ATP 1.6 mM containing CP 1.6 mM.
B. ATP 5 mM containing CP 10 mM.

tributes 70.6% and unlabeled (PC derived) ATP contributes 29.4% to the net P_i formed; at the end of one minute the ATP contains 93.1% of the original label. Thus, when the substrate, labeled ATP, is diluted only to the extent of 7%, the product, P_i, contains a disproportionally high percentage of unlabeled P_i. We interpret this finding to mean that the ATPase and CPK are spatially coupled so that the energy-rich phosphate is efficiently transferred by CPK to ADP formed by the ATPase; the newly formed ATP is compartmentalized in such a way that it gains rapid access to the active site of ATPase at the expense of larger concentrations of ATP in the medium.

In light of the ten fold difference in kinetic constants for the ATPase and CPK reactions, it may be predicted that changes in the levels of the two substrates employed in the coupled reaction system will have an effect on the relative contributions from the two ATP pools. Figure 1 shows the changes in ATP, CP, and P_i when the 1.6 mM ATP plus 1.6 mM CP system is compared to one in which levels of ATP and CP thought to be present in heart cells (5 mM ATP, 10 mM PC) are present (14). $MgCl_2$ concentration has been increased to 5 mM for the latter. The larger concentration of PC can maintain the concentration of ATP at the original level (Fig. 1B). The decrease in the concentration of

PC with time can account for the increase in P_i from the hydrolysis of ATP. On the other hand, in Fig. 1A, the lower concentration of PC cannot maintain ATP at a constant level; the sum of the decrease in the concentrations of PC and ATP yield the amount of P_i observed to increase during the same measured interval. When the SA data are analyzed to obtain % Labeled and % Unlabeled P_i for the one minute interval as described above, the higher concentration of labeled ATP contributes 80% and the higher PC concentration contributes 20% to the net P_i formed, as compared to the average values for the lower concentration system shown in Table II. The SA of ATP in the high concentration system is not diluted to any appreciable extent. Thus at higher concentrations of ATP and PC, the P_i released also contains a disproportionally high percentage of unlabeled P_i.

These experiments indicate that ATPase and myofibrillar CPK have a close functional relationship in that ATP generated from PC via CPK has special access to the myofibrillar ATPase. The preferred hydrolysis of the ATP newly formed from PC adds more weight to the role of PC as an energy carrier. They also provide an explanation for the observation of Perry (15), that in the presence of crude creatine kinase and PC, 3 μM of ADP caused a shortening of glycerinated myofibrils, whereas a similar degree of shortening could not be observed unless more than 60 μM ATP was added directly to the glycerinated myofibrils.

In summary these experiments provide evidence for a myofibrillar end of a creatine-creatine phosphate shuttle system of energy transport between mitochondrion and myofibril.

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