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PHOSPHOCREATINE PRODUCTION COUPLED TO THE GLYCOLYTIC REACTIONS IN THE CYTOSOL OF CARDIAC CELLS

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Summary

Phosphocreatine production catalyzed by a cytosolic fraction from cardiac muscle containing all glycolytic enzymes and creatine kinase in a soluble form has been studied in the presence of creatine, adenine nucleotides and different glycolytic intermediates as substrates. Glycolytic depletion of glucose, fructose 1,6-bis(phosphate) and phospho*enol*pyruvate to lactate was coupled to efficient phosphocreatine production. The molar ratio of phosphocreatine to lactate produced was close to 2.0 when fructose 1,6-bis(phosphate) was used as substrate and 1.0 with phospho*enol*pyruvate. In these processes the creatine kinase reaction was not the rate-limiting step: the mass action ratio of the creatine kinase reaction was very close to its equilibrium value and the maximal rate of the forward creatine kinase reaction exceeded that of glycolytic flux by about 6-fold when fructose 1,6-bis(phosphate) was used as a substrate. Therefore, the creatine kinase raction was continuously in the state of quasi-equilibrium and the efficient synthesis of phosphocreatine observed is a result of constant removal of ADP by the glycolytic system at an almost unchanged level of ATP ([ATP] >> [ADP]), this leading to a continuous shift of the creatine kinase equilibrium position.

When phosphocreatine was added initially at concentrations of 5—15 mM the rate of the coupled creatine kinase and glycolytic reactions was very significantly inhibited due to a sharp decrease in the steady-state concentration of ADP. Therefore, under conditions of effective phosphocreatine production in heart mitochondria, which maintain a high phosphocreatine: creatine ratio in the myoplasm in vivo, the glycolytic flux may be suppressed due to limited availability of ADP restricted by the creatine kinase system. The possible physiological role of the control of the glycolytic flux by the creatine kinase system is discussed.

Introduction

Experimental data collected in several laboratories in recent years demonstrate the important role of the creatine kinase isoenzymes in the intracellular energy transfer from mitochondria to myofibrils and subcellular membrane systems in muscle cells [1—8]. The mitochondrial creatine kinase isoenzyme localized on the outer side of the inner mitochondrial membrane has been shown to catalyze effective phosphocreatine production from mitochondrial ATP and cytosolic creatine [1—4]. The MM isoenzymes of creatine kinase bound to myofibrils, to the membrane of sarcoplasmic reticulum and to the plasma membrane have been demonstrated to be functionally coupled to the ATPases in these structures and to be able to utilize phosphocreatine for immediate rephosphorylation of ADP produced in the energy-consuming reactions [5—7]. However, a significant amount of creatine kinase has been shown to be present in a soluble form in muscle cell cytoplasm [2]. Its functional role is less well defined. Since the cytoplasmic compartment of muscle cells contains the glycolytic system of anaerobic ATP production vitally important for the cells under several metabolic conditions (anoxia, etc.) [9] it may be supposed that the cytosolic fraction of creatine kinase is involved in the phosphocreatine production from glycolytic ATP. In fact, Scopes [10] has demonstrated effective phosphocreatine production coupled to the glycogen depletion in the system reconstituted from purified glycolytic enzymes and creatine kinase. Earlier, Cori et al. [11] have also observed creatine phosphorylation in the presence of 1,3-diphosphoglycerate and a protein fraction of cytosol extracted from skeletal muscle. However, more detailed information is necessary for a quantitative description of the role of cytoplasmic creatine kinase in cardiac cells. Therefore, the purpose of this work was to study the relationship between the creatine kinase and glycolytic reactions in the cardiac cells. With this aim, the reactions catalyzed by the cardiac cytosolic fraction containing all glycolytic enzymes and soluble creatine kinase were investigated in the presence of added creatine, adenine nucleotides and glycolytic substrates.

Materials and Methods

Extraction of a cytosolic fraction. A cytosolic fraction was extracted from rat hearts. Hearts were washed with homogenization medium and homogenized in a Virtis homogenizer at 12 000 rev./min during 1 min in 3 vols. of a medium containing 0.3 M sucrose, 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM magnesium acetate, 0.33 mM dithiothreitol and 0.1 mM EDTA. The resulting homogenate was centrifuged at 100 000 × g for 40 min. The supernatant obtained was dialyzed for 24 h against a solution containing 10 mM potassium phosphate (pH 7.4), 0.33 M dithiothreitol and 0.1 mM EDTA. Dialyzed solutions were concentrated on an Amicon filter (PM 10) to a protein concentration of 40—60 mg per ml and stored at —10 to —15° C.

Incubations. The reactions studied were carried out at 30° C in the medium containing 40—50 mM Tris-HCl, 20 mM potassium phosphate (pH 7.5), 50 mM KCl, 5 mM magnesium acetate, 0.33 mM dithiothreitol and 0.1—20 mg per ml of the cytosolic fraction protein. The addition of substrates and cofactors was made as described in the legends to the figures. At zero time and after definite time intervals shown in the figures, samples of 0.5 ml were withdrawn and

mixed with 0.5 ml cold (0°C) 10% perchloric acid. The mixture was kept for 15 min at 0°C and denaturated protein was removed by filtration through Gelman filters with a pore diameter of 0.45 μm. 0.5 ml of the filtrate was neutralized by the addition of 1.0 ml of 0.3 M Tris (pH approx. 10) containing 1 M KCl. The $KClO_4$ deposit formed was removed by centrifugation and the composition of the supernatant obtained was analyzed by means of methods described below.

Determinations. The concentrations of ATP, ADP, AMP, phosphocreatine and lactate in filtrates were determined by using enzymatic methods. ADP concentrations were assayed by using pyruvate kinase, lactate dehydrogenase, phospho*enol*pyruvate and NADH and recording a decline in the absorbance at 340 nm [12]. For AMP determinations the same system was used; in this case after termination of the reaction with ADP, myokinase (1 I.U./ml) was added and additional changes in the absorbance were recorded. ATP and phosphocreatine concentrations were measured by the hexokinase-glucose-6-phosphate dehydrogenase system and an increase in absorbance at 340 nm due to NADP reduction was recorded [12]. The assay system contained 10 mM AMP to inhibit myokinase contaminations in the reaction mixture. Phosphocreatine concentration was assayed in the same system after ADP and creatine kinase addition as described [12]. Lactate concentration was assayed by the reverse lactate dehydrogenase reaction system in the presence of NAD^+ (2 mM) and hydrazine (0.14 M) at pH 9.0 [13]. Changes in NADH concentration in the reaction medium during incubations were determined in parallel experiments by recording the absorbance at 340 nm using spectrophotometric cells with optical paths of 1 and 10 mm.

Spectrophotometric determination of kinetic constants of creatine kinase and pyruvate kinase. The maximal velocity of the pyruvate kinase present in a cytosolic fraction was found from experimental dependencies of $1/v$ upon 1/[ADP] at 2.0 mM phospho*enol*pyruvate in the presence of 6.0 mM Mg^{2+}, 20 mM creatine and excess of the added lactate dehydrogenase and creatine kinase. The value of the maximal rate of the forward creatine kinase reaction of a cytosolic fraction was determined from dependencies of $1/v$ upon 1/[ATP] in the presence of 20 mM creatine and excess of added pyruvate kinase and lactate dehydrogenase, extrapolating values obtained to infinite creatine were purchased from Sigma and Calbiochem (U.S.A.), lactate dehydromined for every cytosol preparation.

Reagents. Pyruvate kinase, hexokinase, glucose-6-phosphate dehydrogenase, creatine kinase, myokinase, dithiothreitol, phospho*enol*pyruvate, fructose 1,6-bis(phosphate) tetrasodium salt, all nucleotides, creatine and phosphocreatine were purchased from Sigma and Calbiochem (U.S.A.), lactate dehydrogenase from Reanal (Hungary).

Spectrophotometric determinations were carried out in Aminco DW-2 UV-VIS (U.S.A.) and Perkin Elmer Model 402 (U.S.A.) spectrophotometers.

Results

Phosphocreatine production coupled to glycolytic depletion of glucose, fructose 1,6-bis(phosphate) and phosphoenolpyruvate

The experimental data presented in Fig. 1 demonstrate that a cytosolic frac-

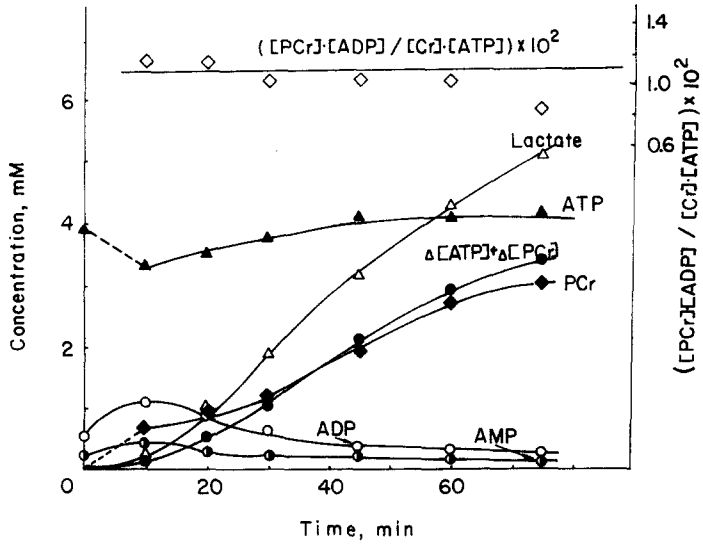

Fig. 1. Phosphocreatine formation coupled to glycolytic cleavage of glucose. Ordinate (right-hand side) of upper horizontal line corresponds to the value of the apparent equilibrium constant for the creatine kinase reaction under these experimental conditions ($1.1 \cdot 10^{-2}$). The incubation medium contained 50 mM glucose, 3.9 mM ATP, 0.5 mM ADP, 0.2 mM AMP, 0.5 mM NAD^+, 20 mM creatine and 1.1 mg/ml of cytosolic protein. PCr, phosphocreatine; Cr, creatine.

tion extracted from rat heart is able to catalyze the depletion of glucose to lactate; in the presence of creatine the glycolysis is coupled to phosphocreatine synthesis. The reactions were initiated by the addition of ATP (4 mM). After some lag-phase the lactate and phosphocreatine concentrations were increased almost linearly with time. Obviously, the initial drop in ATP concentration is a result of glucose, fructose 6-phosphate and creatine phosphorylation; during these initial reactions lactate is not produced. Then the ATP level is restored due to its production in the glycolytic system and continuous phosphocreatine

TABLE I

CHANGES IN PHOSPHOCREATINE, ATP AND ADP CONCENTRATIONS AND OF THE MASS-ACTION RATIO FOR THE CREATINE KINASE REACTION DURING GLYCOLYTIC CONVERSION OF FRUCTOSE 1,6-BIS(PHOSPHATE), CATALYZED BY THE CYTOSOLIC FRACTION FROM CARDIAC CELLS

The incubation medium initially contained 5 mM fructose 1,6-bis(phosphate), 20 mM creatine, 3.6 mM ADP, 1.1 mM AMP, 0.13 mM ATP, 0.5 mM NAD and 1.1 mg/ml of cytosolic protein. Temperature 30°C, pH 7.5. PCr, phosphocreatine; Cr, creatine.

Parameter	Time of incubation (min)			
	10	15	20	30
[PCr] (mM)	0.45	0.98	2.60	5.23
[ATP] (mM)	2.52	3.43	4.11	4.18
[ADP] (mM)	1.29	0.74	0.28	0.13
$\frac{[ADP] \cdot [PCr]}{[ATP] \cdot [Cr]}$ ($\times 10^2$)	1.18	1.11	1.02	0.96

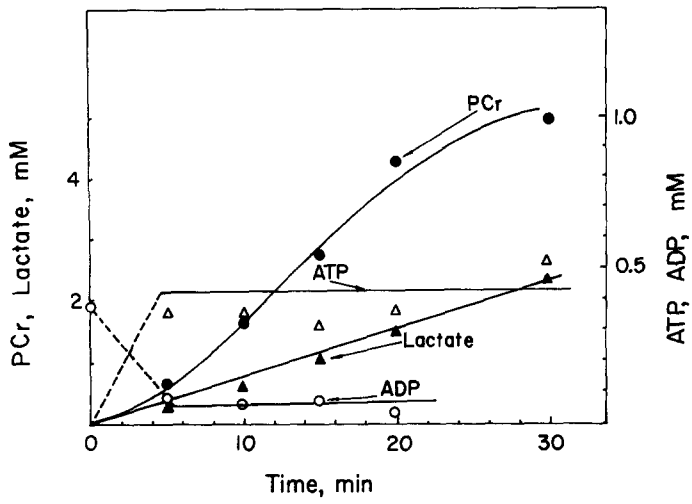

Fig. 2. Phosphocreatine (PCr) synthesis coupled to glycolytic splitting of fructose 1,6-bis(phosphate). The incubation medium contained 5 mM fructose 1,6-bis(phosphate), 0.4 mM ADP, 0.5 mM NAD, 20 mM creatine and 1.1 mg/ml of cytosolic protein.

synthesis is observed. It can be seen from Fig. 1 that the ratio, (Δ[ATP] + Δ[phosphocreatine])/[lactate], was close to 1.0 for the maximal slopes of curves. Some delay in high-energy phosphate production as compared to that of lactate can be explained by non-steady-state conditions, the rate of phosphorylation of hexoses probably exceeding half of the rate of high-energy phosphate production.

The mass-action ratio for the creatine kinase reaction, [phosphocreatine] × [ADP]/[creatine] × [ATP], calculated from the experimental data did not change with the time of reaction and was very close to the apparent equilibrium constant of the reaction under the experimental conditions used (K_{eq} = $1.1 \cdot 10^{-2}$ as determined by using a purified creatine kinase and measuring the equilibrium concentrations of ATP, ADP, phosphocreatine and creatine when they reached a constant value in the mixture).

Therefore, it can be concluded that the creatine kinase reaction was not the rate-determining step in the phosphocreatine synthesis.

Fig. 2 shows phosphocreatine production coupled to the glycolytic depletion of fructose 1,6-bis(phosphate). The reaction was initiated by addition of ADP at a low concentration (0.38 mM). ADP added was immediately phosphorylated and phosphocreatine and lactate production were observed without delay at a constant ATP level (0.35 mM). The stoicheiometric ratio, [phosphocreatine]/[lactate], produced was equal to 2.0 as can be theoretically predicted.

The steady-state rate of phosphocreatine production as determined from the linear part of the curve, Fig. 2, is dependent on the concentration of ATP in the system. In Lineweaver-Burk plots this dependence gives a straight line and a corresponding apparent K_m value for ATP equal to 0.25 mM and V = 0.4 μmol/min per mg (Fig. 3B). These constants characterize the overall phosphocreatine-production process. The rate of ATP synthesis coupled to glycolytic conversion

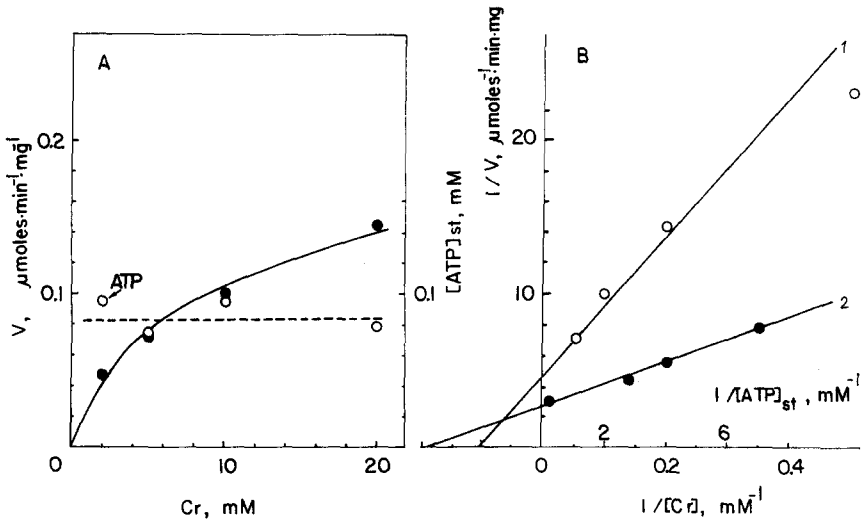

Fig. 3. (A) The dependence of the rate of phosphocreatine production coupled to fructose 1,6-bis(phosphate) splitting on the creatine (Cr) concentration. The incubation medium initially contained 80 μM ADP and 1 mg/ml of cytosolic protein. Other experimental conditions were as in the legend to Fig. 2. The dotted line shows changes in the ATP concentration. (B) Dependence of reciprocal rate of phosphocreatine formation coupled to fructose 1,6-bis(phosphate) splitting on reciprocal concentration of creatine (line 1, $[ADP]_o$ = 80 μM) and reciprocal steady-state ATP concentration (line 2, $[Cr]_o$ = 20 mM). Conditions of the experiments were the same as given in the legend to Fig. 2, the only exception was that in this experiment concentrations of ADP or creatine added were varied.

of fructose 1,6-bis(phosphate) in the absence of creatine and at a high concentration of ADP (4 mM) was very close to the maximal rate of phosphocreatine production in this system. On the other hand, cytosolic creatine kinase itself is characterized by the apparent K_m value for ATP equal to 0.8 mM and V = 2.5 μmol/min per mg. Such a ratio of V values leads to the conclusion that the glycolytic reactions are the rate-limiting steps.

This conclusion is confirmed by the constant value of the mass-action ratio of the creatine kinase reaction in the system studied with fructose 1,6-bis(phosphate) as a substrate (Table I). This ratio was in the range $0.96—1.2 \cdot 10^{-2}$ and very close to the apparent equilibrium constant ($K_{eq} = 1.1 \cdot 10^{-2}$), as it was with glucose as a substrate (Fig. 1) even at a significantly higher glycolytic flux from fructose 1,6-bis(phosphate) to lactate (0.4 μmol/min per mg compared to 0.1 μmol/min per mg with glucose).

Fig. 3A shows also the influence of creatine on the phosphocreatine-production rate in the presence of fructose 1,6-bis(phosphate) at low added ADP concentration (0.1 mM). Linearization of this dependence in double-reciprocal plots gives a value of K_m for creatine about 10 mM, close to that for creatine kinase itself (Fig. 3B). These results mean that creatine can efficiently control the phosphocreatine-production rate in spite of the fact that the creatine kinase reaction is not the rate-limiting step. This regulatory mechanism most probably consists of increasing steady-state concentration of ADP with elevation of creatine concentration at an almost constant ATP level (see Fig. 3A) under conditions when $[ATP]_{st.st} >> [ADP]_{st.st}$ (prefix, st.st., refers to steady state).

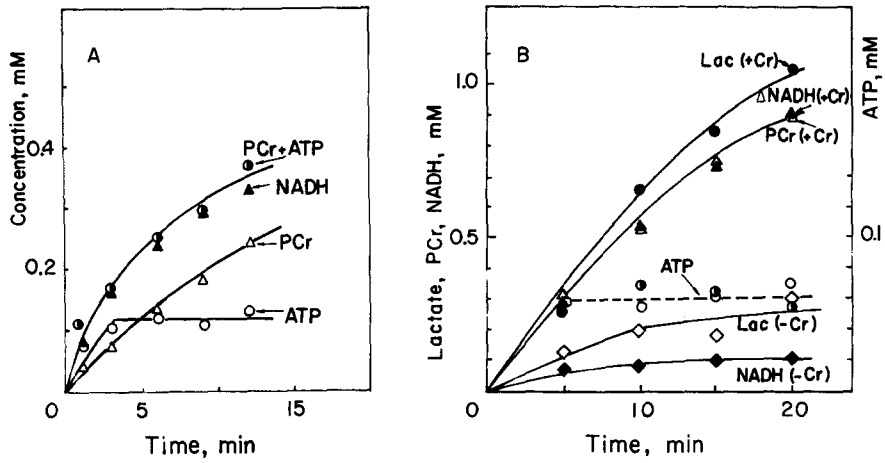

Fig. 4. (A) Phosphocreatine (PCr) production coupled to the phosphoglycerate kinase reaction. 5.0 mM fructose 1,6-bis(phosphate), 2.0 mM NAD, 20 mM creatine (Cr), 0.12 mM ADP, 2.0 I.U./ml of glyceraldehyde-3-phosphate dehydrogenase, as well as 20 mM NaF and 0.2 mg/ml of cytosolic protein were added into the medium. (B) Phosphocreatine synthesis coupled to glycolytic cleavage of phospho*enol*pyruvate. Dotted line shows steady-state ATP concentration with creatine (●) and without creatine (○) in the incubation mixture. The incubation medium contained 3 mM phospho*enol*pyruvate, 50 μM ADP, 2.0 mM NADH, 20 mM NaF, 20 mM creatine where indicated in the figure and 1.0 mg/ml of cytosolic protein.

With fructose 1,6-bis(phosphate) as a substrate, phosphocreatine production results from the coupling of the creatine kinase reaction with 3-phosphoglycerate kinase and pyruvate kinase reactions. When the enolase reaction is inhibited by 20 mM NaF [14], and fructose 1,6-bis(phosphate) (5 mM) and glyceraldehyde-3-phosphate dehydrogenase (2 I.U./ml) are used as the 1,3-diphosphoglycerate-generating system, the coupling of phosphocreatine production to the phosphoglycerate kinase reaction can be demonstrated separately as is shown in Fig. 4A. The amount of NADH formed due to glyceraldehyde 3-phosphate oxidation to 1,3-diphosphoglycerate is very close to the amount of high-energy phosphates produced in the subsequent phosphoglycerate and creatine kinase reactions. Since ATP concentration is not changed remarkably, the stoicheiometric coefficient in this case is close to 1 (theoretically maximal value).

When phospho*enol*pyruvate is used as a substrate, phosphocreatine production coupled to the pyruvate kinase reaction in a cytosolic fraction can be also easily observed in the presence of NaF. In this case, NaF is necessary to inhibit the utilization of phospho*enol*pyruvate in the reverse glycolytic reactions producing 3-phosphoglycerate which in turn may be phosphorylated to 1,3-diphosphoglycerate at the expense of ATP (creatine and pyruvate kinases activities were not changed by NaF). In this case, as is shown in Fig. 4B, in the presence of creatine formation of 1 mol of lactate is accompanied by oxidation of 1 mol of NADH and 1 mol of phosphocreatine is produced at a constant level of ATP.

The experimental results described show the efficient phosphocreatine production coupled to the glycolytic reactions in the heart cell cytosol with

stoicheiometric coefficients close to the theoretical ones. Given below is the quantitative analysis of phosphocreatine synthesis coupled to glycolysis, which shows that this process can be completely described by the kinetics of the reactions in a homogeneous medium. The pyruvate kinase step of glycolytic ATP production was analyzed with this purpose in mind.

Mathematical simulation of the coupled pyruvate kinase-creatine kinase reactions and the influence of phosphocreatine

The rate of the pyruvate kinase reaction, v_{PK}, and that of the creatine kinase reaction, v_{CK}, are described by the following equations [1,15]:

$$v_{PK} = (V_2[PEP][Mg^{2+}][MgADP]/K_A K'_m)/(K_m^{PEP} + [PEP])$$
$$\times\left(\left[1 + \frac{[Mg^{2+}]}{K_A} + \left(1 + \frac{[Mg^{2+}]}{K'_A}\right)\frac{[MgADP]}{K_m} + \left(1 + \frac{[Mg^{2+}]}{K''_A}\right)\frac{[ADP]}{K_I}\right]\right.$$
$$\left. + \left(1 + \frac{[Mg^{2+}]}{K_A}\right)\frac{[MgATP]}{K_I^{ATP}}K_m^{PEP}\right)^{-1} \quad (1)$$

$$v_{CK} = \left(V_1\frac{[MgATP][Cr]}{K_{ia}K_b} - V_{-1}\frac{[MgADP][PCr]}{K_{ic}K_d}\right)$$
$$\times\left(1 + \frac{[Cr]}{K_{ib}} + \frac{[PCr]}{K_{id}} + \frac{[MgATP]}{K_{ia}}\left(1 + \frac{[Cr]}{K_b}\right)\right.$$
$$\left. + \frac{[MgADP]}{K_{ic}}\left(1 + \frac{[PCr]}{K_d} + \frac{[Cr]}{K_{ib}}\right)\right)^{-1} \quad (2)$$

where K_A, K'_A, K''_A are the dissociation constants for Mg^{2+} from the enzyme-substrate complexes of pyruvate kinase: $E \cdot Mg^{2+}$, $E \cdot MgADP \cdot Mg^{2+}$ and $E \cdot Mg^{2+} \cdot ADP$, respectively, K_m^{PEP} is the Michaelis constant for phospho*enol*-pyruvate (PEP), K_m and K'_m are the dissociation constants for MgADP from complexes $E \cdot MgADP$ and $Mg^{2+} \cdot E \cdot MgADP$, respectively, K_I^{ATP} is the inhibition constant of the pyruvate kinase by MgATP, V_2 the maximal rate of the pyruvate kinase reaction [15]; K_{ia}, K_{ib}, K_{ic} and K_{id} are the dissociation constants for MgATP, creatine (Cr), MgADP and phosphocreatine (PCr) from their complexes with creatine kinase; K_a, K_b, K_c and K_d are the same constants for dissociation of these substrates from their ternary complexes with creatine kinase containing two substrates; and V_1 and V_{-1} are the maximal rates of the forward and reverse creatine kinase reactions, respectively [1].

These two equations were used for simulation of the steady-state rates of phosphocreatine production; under steady-state conditions, v_{CK} was taken to be equal to v_{PK} and Eqns. 1 and 2 were solved to find the steady-state concentration of ADP and ATP and the numerical values of the rates for given conditions of the reaction. In these calculations the kinetic constants given in Table II were used and the concentrations of Mg complexes of adenine nucleotides and phosphate were calculated as described earlier, using the stability constants obtained on the basis of Phillips' equations [18] for ionic strength of 0.16–0.25 M. The calculations were carried out with a Hewlett Packard minicomputer, and the calculated values of the rates were compared with the experimental data.

TABLE II

KINETIC PARAMETERS OF MUSCULAR PYRUVATE KINASE AND CARDIAC CREATINE KINASE UNDER EXPERIMENTAL CONDITIONS

K_m^{PEP} for purified muscle pyruvate kinase was determined from the dependence of $1/v$ vs. $1/[PEP]$ at high MgADP concentration equal to 3.0 mM under the conditions used. K_i^{CP} for purified enzyme was determined from the dependencies of $1/v$ vs. [PCr] at different phospho*enol*pyruvate concentrations. Inhibition was competitive with respect to phospho*enol*pyruvate, this is in good accordance with Kemps' data [17]. The inhibition constant of pyruvate kinase by MgATP was taken from Ref. 16. Values of other kinetic constants were taken from our previous papers [1,2,15]. Values of all constants expressed in mM.

Pyruvate kinase (from rabbit muscle)

Constant:	K'_m	K_m^{PEP}	K_A	K'_A	K''_A	K_i^{ADP}	K_i^{CP}	K_i^{MgATP}
	0.25	0.14	0.015	0.047	0.047	2.1	3.0	0.14

Maximal rate: V_2 = 1.6 μmol/min per mg cytosolic protein

Creatine kinase (rat heart)

Constant:	K_a	K_b	K_{ia}	K_{ib}	K_c	K_d	K_{ic}	K_{id}
	1.0	11	1.0	11	0.16	2.0	0.16	2.0

Maximal rates: V_1 = 2.5 μmol/min per mg cytosolic protein
$V_{-1} = 3.5 \times V_1$

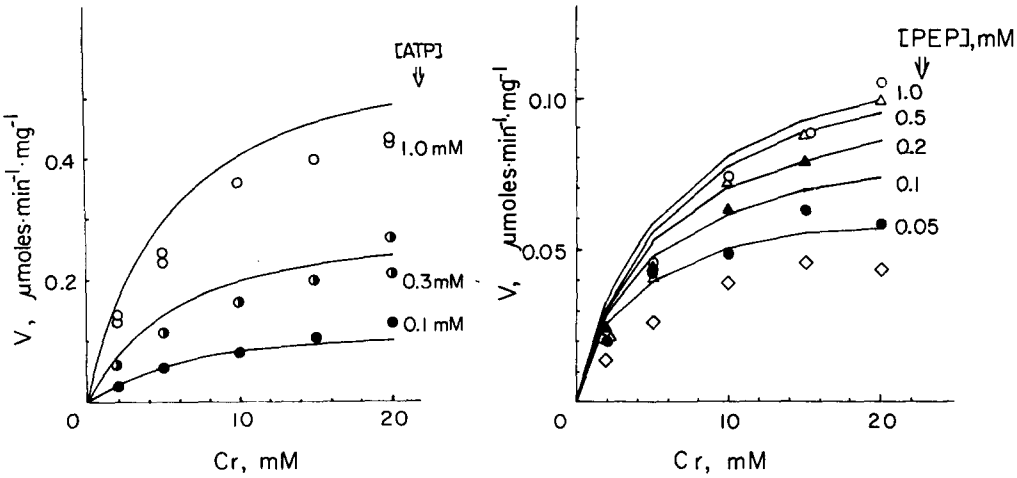

Fig. 5. Dependence of the rates of phosphocreatine synthesis coupled to pyruvate kinase reaction on the initial creatine and ATP concentrations. Curves shown in the figure were calculated on the basis of the kinetic models for creatine kinase and pyruvate kinase, separate symbols are the experimental results. In this and following experiments (Figs. 5—7) the rate of the reaction was measured spectrophotometrically by recording the decrease in absorbance at 340 nm due to a decrease in NADH concentration in the assay medium. Assay medium contained 0.5—1.0 mg/ml of cytosolic protein.

Fig. 6. Dependence of the rate of phosphocreatine formation coupled to pyruvate kinase reaction on creatine concentration at different phospho*enol*pyruvate (PEP) concentrations: 0.05 mM (◇), 0.1 mM (●), 0.2 mM (▲), 0.5 mM (△), 1.0 mM (○). Curves shown in the figure were calculated on the basis of kinetic models for creatine kinase and pyruvate kinase, separate symbols are the experimental results. ATP concentration was 0.1 mM.

Fig. 5 shows the experimental dependencies of v_{st} on the creatine concentration at different ATP concentrations (phospho*enol*pyruvate concentration was high and constant). Separate points presented in this figure are experimental data and solid lines are dependencies calculated by mathematical simulation with use of complete rate equations for creatine and pyruvate kinase (Eqns. 1 and 2).

Fig. 6. shows the dependencies of the reaction rates on the creatine concentration at a fixed level of ATP (0.1 mM) and at different phospho*enol*pyruvate concentrations. Particular points shown in the figure are experimentally determined and solid lines are theoretically predicted on the basis of a mathematical model consisting of Eqns. 1 and 2; a good fitting of theoretical curves with the experimental data can be seen in both Figs. 5 and 6. Thus, the experimental dependencies can be quite precisely described by the system of the two Eqns. 1 and 2 describing homogeneous enzyme kinetics. Initial parts of the curves in Fig. 6 are close to each other in the range of phospho*enol*pyruvate concentration from 0.1 to 1.0 mM ($[PEP]/K_m^{PEP}$ changes from 0.7 to 7.0) if creatine concentration is lower than 5 mM. At creatine concentrations higher than 5 mM, the curves are separated for 0.05—0.5 mM phospho*enol*pyruvate concentra-

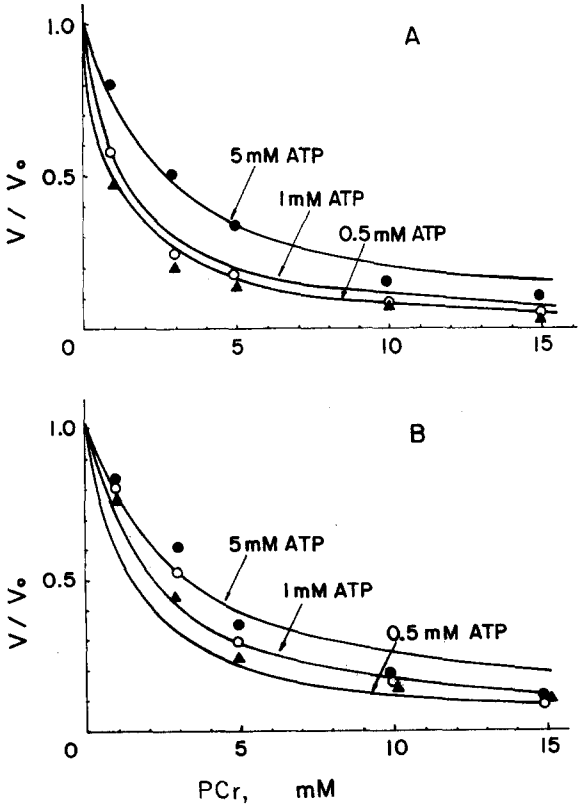

Fig. 7. Inhibition of phosphocreatine (PCr) synthesis coupled to the pyruvate kinase reaction by phosphocreatine at different initial creatine and ATP concentrations. (A) 10 mM creatine, (B) 20 mM creatine. Curves given in the figure were calculated on the basis of kinetic models for creatine and pyruvate kinases. Concentration of cytosolic protein was in the range 0.1—0.8 mg/ml.

tions. Apparently, at creatine concentrations lower than 5 mM, the creatine kinase reaction is the rate-limiting step. At higher creatine concentrations, the rate-limiting step is shifted onto the pyruvate kinase reaction and the whole system becomes sensitive to phospho*enol*pyruvate concentration.

One of the most important questions with regard to the coupled glycolytic and creatine kinase reactions is the sensitivity of such a system to product inhibition by phosphocreatine which in vivo heart cells is present at a high concentration (15—20 mM). Therefore, we have investigated the effect of phosphocreatine on the steady-state rate of the coupled pyruvate kinase-creatine kinase reactions both theoretically and experimentally.

The effect of increasing phosphocreatine concentrations on the rate of the coupled reactions at different ATP and creatine concentrations is shown in Fig. 7A and B. It can be seen from these data that an increase in the phosphocreatine concentration up to 10—15 mM leads to a strong inhibition of the reactions: in the presence of 15 mM phosphocreatine the rates of coupled pyruvate and creatine kinase reactions are equal to approx. 10% of their initial values. The separate dots in Fig. 7A and B show the experimentally determined relative reaction rates, the solid curves show the calculated dependencies obtained by mathematical simulation using the complete rate Eqns. 1 and 2. A good fitting between calculated and experimental reaction rates can be seen from Fig. 7A and B.

Discussion

The results of this study show that the cytosolic fraction from rat cardiac muscle containing all glycolytic enzymes and creatine kinase probably in proportions close to those in vivo catalyzes efficient synthesis of phosphocreatine in the presence of creatine with glucose, fructose 1,6-bis(phosphate) and phospho*enol*pyruvate as substrates. The stoicheiometric ratios of phosphocreatine to lactate produced were in all cases close to the theoretically maximal ones. At sufficiently high creatine concentrations the creatine kinase reaction was not the rate-determining step, that being located in the glycolytic pathway. This conclusion emerges from the value of the creatine kinase mass-action ratio being constant and equal to the apparent equilibrium constant of the reaction under the conditions used. The maximal rate of the creatine kinase reaction also significantly exceeds the observed values of the glycolytic fluxes.

The efficient phosphocreatine production can be explained by a shift of the creatine kinase equilibrium position by the glycolytic reactions which continuously remove ADP, decreasing its concentration. For such conditions, the following expression can be used:

$$[PCr]_t = \frac{[ADN]_0}{[ADP]_t} \cdot\cdot [Cr] \cdot K_{eq}^{CK}$$

where $[ADN]_0$ is the total concentration of adenine nucleotides added, that is close to the ATP steady-state concentration, $[PCr]_t$ and $[Cr]_t$ are the phosphocreatine and creatine concentrations at a given time of the reaction, respectively, and K_{eq}^{CK} is an apparent equilibrium constant of the creatine kinase reaction.

It should be mentioned that in the absence of the phosphocreatine-consuming processes, the real steady state of the system cannot be reached since the ADP concentration continuously decreases with the time of the reaction. The maximal concentration of phosphocreatine which can be synthesized at a given creatine concentration depends upon the thermodynamics of the whole system and is determined by the nature of the glycolytic substrate used.

The results of a kinetic analysis of the coupled creatine kinase and pyruvate kinase reactions also conform to the quasi-equilibrium state of the creatine kinase reaction. The behaviour of this two-enzyme system can be quantitatively described by the kinetic equations for these individual enzymes interacting in the homogeneous medium. In such a system, an increase in phosphocreatine concentration leads to a strong inhibition of the coupled reactions as a result of a decrease in available ADP concentration for the pyruvate kinase reaction (as well as for other glycolytic reactions). Moreover, the concentration of another important regulator of phosphofructokinase, AMP, may also be related to the phosphocreatine concentration through the creatine kinase and adenylate kinase equilibrium according to the following relationship:

$$\frac{[Cr]}{[PCr]} \cdot K_{eq}^{CK} \simeq \frac{[ADP]}{[ATP]} \simeq \frac{[AMP]}{[ADP]} \cdot K_{eq}^{AK}$$

where K_{eq}^{AK} is the adenylate kinase reaction equilibrium constant. Therefore, a decrease in phosphocreatine concentration under anoxic or hypoxic conditions [19—21] should lead to a proportional increase in the ADP concentration and to a sharper increase in AMP concentration (proportional to $[ADP]^2$), which activates the phosphofructokinase reaction and increases the glycolytic flux under these conditions, and which is in good agreement with many observations [19—21].

Besides the regulatory action of phosphocreatine on the glycolytic flux via ADP and AMP concentrations, the glycolytic system may be affected by phosphocreatine due to its direct inhibitory action on some glycolytic key enzymes such as phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase [17,22—26]. In the experiments described, the concentration of phospho*enol*pyruvate was high (2.0 mM) and protected the pyruvate kinase reaction from significant inhibition due to a direct binding of phosphocreatine and ATP with the enzyme which are competitive inhibitors with respect to phospho*enol*pyruvate [16,17]. However, under physiological conditions, phospho*enol*pyruvate concentration is more than an order of magnitude lower [9] and the direct inhibitory effect of phosphocreatine on the pyruvate kinase reaction may be stronger.

Since the values of the glycolytic flux in vivo are much lower than the maximal activities of the individual enzymes, including creatine kinase [27], the results of this study may be of importance in understanding some metabolic events occurring in cardiac cells in vivo. In this case, the results obtained conform to the conclusion made by many investigators that in muscle cells the creatine kinase reaction is in a state of quasi-equilibrium [28—30]. However, this conclusion can be valid only if the cytoplasmic compartment of the cells is considered or if the glycolytic reactions are the main source of available energy for the cells (low respiratory rates at low work loads, or under anoxic

conditions) [31—34]. On the other hand, at high rates of oxygen consumption and oxidative phosphorylation characteristic of working caridac muscle [27,29], the main amount of energy is produced in mitochondria. Specific localization of mitochondrial creatine kinase on the outer side of the inner mitochondrial membrane results in significant acceleration of the forward creatine kinase reaction and phosphocreatine production by oxidative phosphorylation [1,8]. A peculiar way of mitochondrial creatine kinase interaction with oxidative phosphorylation and most probably with ATP-ADP translocase as proposed [1,8] shifts the mitochondrial enzyme from its equilibrium with cytoplasmic substrates, and its functioning in the steady state obviously cannot be explained by a simple equation for creatine kinase equilibrium used, for example, by Kohn et al. [30].

Considering the cytoplasmic compartment, it should be mentioned that the glycolytic enzymes as well as cytoplasmic creatine kinase may not exist in the freely soluble form but may be associated with different cellular structures [35—42], which usually results in changes of their kinetic properties [35,36,38—40]. The possibility of glycolytic enzymes existence in a structurally organized state should be obviously accounted for in the quantitative description of their behaviour in vivo.

Thus, if the intracellular phosphocreatine concentration is kept high and constant due to its efficient production in mitochondria coupled to the oxidative phosphorylation, the glycolytic flux should be significantly inhibited. Such an influence of phosphocreatine may contribute to the control of glycolysis exerted by the reactions of fatty acid oxidation under aerobic conditions in cardiac cells [27].

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