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Velocity of the Creatine Kinase Reaction in the Neonatal Rabbit Heart: Role of Mitochondrial Creatine Kinase†

Stanton B. Perry,* John McAuliffe,‡ James A. Balschi, Paul R. Hickey, and Joanne S. Ingwall
Harvard Medical School NMR Laboratory, Departments of Cardiology and Anesthesiology, The Children's Hospital, and Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115
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ABSTRACT: To examine the role of changes in the distribution of the creatine kinase (CK) isoenzymes [BB, MB, MM, and mitochondrial CK (mito-CK)] on the creatine kinase reaction velocity in the intact heart, we measured the creatine kinase reaction velocity and substrate concentrations in hearts from neonatal rabbits at different stages of development. Between 3 and 18 days postpartum, total creatine kinase activity did not change, but the isoenzyme distribution and total creatine content changed. Hearts containing 0, 4, or 9% mito-CK activity were studied at three levels of cardiac performance: KCl arrest and Langendorff and isovolumic beating. The creatine kinase reaction velocity in the direction of MgATP production was measured with ^{31}P magnetization transfer under steady-state conditions. Substrate concentrations were measured with ^{31}P NMR (ATP and creatine phosphate) and conventional biochemical analysis (creatine) or estimated (ADP) by assuming creatine kinase equilibrium. The rate of ATP synthesis by oxidative phosphorylation was estimated with oxygen consumption measurements. These results define three relationships. First, the creatine kinase reaction velocity increased as mito-CK activity increased, suggesting that isoenzyme localization can alter reaction velocity. Second, the reaction velocity increased as the rate of ATP synthesis increased. Third, as predicted by the rate equation, reaction velocity increased with the 3-fold increase in creatine and creatine phosphate contents that occurred during development.

Creatine kinase (EC 2.7.3.2, creatine phosphotransferase), present in high activity in heart, catalyzes the reaction MgADP + creatine phosphate ↔ MgATP + creatine. In heart, there are four creatine kinase isoenzymes: MM, MB, BB, and mitochondrial (mito-CK).[1] Mito-CK, bound on the inner membrane of the mitochondria, and MM-CK, a significant portion of which is localized at sites of ATP utilization, are the predominant isoenzymes in mature myocardium. MB- and BB-CK, which are cytosolic enzymes, are present primarily in immature myocardium.

Controversy persists regarding the role of the creatine kinase in energy transfer in muscle and the determinants of the creatine kinase reaction velocity in vivo. To date, studies using ^{31}P magnetization transfer to measure the creatine kinase reaction velocity in intact hearts have primarily examined the role of substrates in regulating the reaction velocity. While it is accepted that the creatine kinase reaction velocity is severalfold faster than the rate of ATP synthesis by oxidative phosphorylation, the relationship between changes in the rate of ATP synthesis and the creatine kinase reaction velocity remains controversial. Studies looking at wide ranges of cardiac performance, including arrest (Kupriyanov et al., 1984; Bittl & Ingwall, 1985, 1986), have shown that the reaction

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* Address correspondence to this author at the Harvard Medical School NMR Laboratory, Room 209, 221 Longwood Ave., Boston, MA 02115.
‡ Present address: Department of Anesthesia, Children's Hospital Medical Center, Eland and Bethesda Ave., Cincinnati, OH 45229.

[1] Abbreviations: CK, creatine kinase; CP, creatine phosphate; mito-CK, mitochondrial creatine kinase; Na_2H_2EDTA, disodium ethylenediaminetetraacetate; T_1, longitudinal nuclear relaxation time; RPP, rate-pressure product (product of heart rate times left ventricular developed pressure).

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velocity increases as the rate of ATP synthesis increases despite only small changes in steady-state substrate concentrations. The hyperbolic nature of the curve relating the rate of ATP synthesis via the creatine kinase reaction and by oxidative phosphorylation may explain the apparently contradictory results of studies (Matthews et al., 1982; Shoubridge et al., 1985) that show no change in the creatine kinase reaction velocity over narrower ranges of cardiac performance. Taking a different approach, Shoubridge et al. (1985) studied hearts from rats fed a creatine analogue, β-guanidinopropionic acid, that accumulates in muscle in preference to creatine. They showed that, at constant levels of cardiac performance, decreases in the creatine pool were associated with decreases in the reaction velocity; however, the effect, if any, of replacing the creatine with β-guanidinopropionic acid on creatine kinase activity and isoenzyme distribution was not reported. Taken together, these results suggest that the creatine kinase reaction velocity changes with the size of substrate pools at constant rates of ATP synthesis and with the rate of ATP synthesis at relatively constant cytosolic substrate concentrations.

The relationship between creatine kinase isoenzyme distribution and the creatine kinase reaction velocity in the intact heart remains undefined. A study of spontaneously hypertensive rats (Ingwall, 1984) reported preliminary data suggesting that changes in the isoenzyme distribution, including a decrease in both MM- and mito-CK (and hence total) activity, were associated with decreases in the reaction velocity at constant levels of cardiac performance. The potential importance of the localization of isoenzymes on the magnitude of the reaction velocity has been demonstrated in a series of in vitro experiments using mitochondrial, sarcolemmal, and myofibrillar preparations (Saks et al., 1976, 1984, 1985; Saks & Kupriyanov, 1982). These studies are consistent with functional coupling between mito-CK and the adenine nucleotide translocase at the mitochondria and between MM-CK and ATPases at sites of ATP utilization. These results constitute the major source of support for the proposed role of the creatine kinase system as an energy shuttle in which creatine phosphate is produced at the mitochondria by mito-CK and ATP is resynthesized at sites of ATP utilization by MM-CK. An analysis based on facilitated diffusion (Meyer et al., 1984) demonstrated that the creatine kinase system operating at near equilibrium would serve both as a high-energy phosphate buffer and as an energy transporter. While distribution or localization of isoenzymes is not a critical component of this analysis, the model does allow for the possibility that localizing the isoenzymes could alter the proportion of high-energy phosphates transported as creatine phosphate. To test this, the relationship between creatine kinase isoenzyme distribution and the creatine kinase reaction velocity in the intact heart must be defined.

To address this issue, we took advantage of changes in the creatine kinase isoenzyme distribution that occur during neonatal development in rabbits: the proportion of BB-CK and MB-CK isoenzymes decreases while the proportion of MM-CK and mito-CK isoenzymes increases. In the present study, neonatal rabbit hearts at different stages of development were studied over a range of cardiac performance. Hearts were analyzed in terms of total creatine kinase activity, creatine kinase isoenzyme distribution, steady-state concentrations of substrates of the creatine kinase reaction, oxygen consumption, and the creatine kinase reaction velocity by a combination of ^{31}P NMR and conventional biochemical techniques. By comparing changes in the creatine kinase reaction velocity to changes in isoenzyme distributions (and hence localization of isoenzymes), substrate concentrations, and ATP synthesis rates estimated from oxygen consumption, we found that the creatine kinase reaction velocity changes with mito-CK activity, size of the creatine pool, and rate of ATP synthesis.

Experimental Procedures

Isolated Heart Preparation. Experiments utilized hearts from New Zealand White rabbits which were 3 (group I) or 18 days of age (group II). Following anesthesia with intraperitoneal sodium pentobarbital, the heart was excised, arrested in ice-cold buffer, blotted, and weighed. Hearts to be used for NMR experiments and oxygen consumption measurements were then connected to the aortic cannula of a perfusion apparatus. Retrograde perfusion of the aorta was maintained at a constant flow rate of between 15 and 22 mL min^{-1} (g of tissue)$^{-1}$ with modified Krebs–Henseleit buffer containing 121 mM NaCl, 23 mM $NaHCO_3$, 4.7 mM KCl, 1.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 mM Na_2H_2EDTA, and 11 mM glucose. The perfusate was equilibrated with 95% O_2/5% CO_2, maintaining pH at 7.4. Experiments were performed at 37 °C. Separate hearts were used for measurement of oxygen consumption and for the NMR experiments.

Three levels of cardiac performance were investigated: arrest, achieved by increasing the KCl concentration of the buffer to 20 mM; low workload, achieved by using an empty, beating heart, the Langendorff preparation; and high workload, achieved by using the isovolumic, beating heart obtained by allowing the heart to beat with a left ventricular balloon in place. For these experiments and for the KCl-arrested hearts, a water-filled balloon was inserted and secured across the mitral valve in the left ventricle. Connecting the balloon and aortic cannula to Statham DB23 pressure transducers and a Hewlett-Packard recorder allowed monitoring of the heart rate, ventricular pressure, and perfusion pressure. The volume of the balloon was adjusted to achieve an end diastolic pressure of 0–10 mmHg, and performance was quantified with the rate–pressure product (RPP), that is, the product of heart rate and developed pressure (systolic minus diastolic pressures).

Variation in heart rate, left ventricular pressure, perfusion pressure, ATP or creatine phosphate contents, or pH by more than 10% during an experiment resulted in rejection of the preparation. Hearts were weighed following perfusion and frozen for subsequent biochemical analysis; some hearts were freeze-clamped.

To determine the ratio of wet weight to dry weight, hearts from each group were dried at 50 °C to a constant weight. The ratio was 5.4 and did not vary among groups.

Oxygen Consumption. Oxygen tension was measured in the perfusate at the aortic cannula and in the coronary effluent with a Clark-type electrode (Johnson Foundation Biomedical Laboratories, University of Pennsylvania). Measuring coronary flow allowed calculation of oxygen consumption (Neely et al., 1967). Values for oxygen consumption were converted to ATP synthesis rates by assuming a P:O ratio of 3.

^{31}P NMR. Experiments were carried out in a Nicolet wide-bore NT 360 pulsed Fourier transform NMR spectrometer. At 8.45 T, the phosphorus frequency is 145.75 MHz. A 12-mm horizontal probe was used for 3-day-old hearts and a 20-mm vertical, dual (sodium–phosphorus) probe for 18-day-old hearts. By use of an 18-channel Oxford Instruments Shim Supply, the field was homogenized by maximizing the intensity of the proton signal for the 12-mm probe and the sodium signal for the 20-mm probe. Spectra were obtained by signal-averaging 128–256 scans of a 60° broad-band pulse.

^{31}P NMR spectroscopy was used to define the steady-state concentrations of creatine phosphate and ATP. To determine

the concentration of ATP in the tissue, spectra of known amounts of ATP were obtained and compared with the areas under the ATP β-P peaks from the hearts. Intracellular pH was determined from standards with the chemical shift of inorganic phosphate.

Magnetization transfer was used to study the creatine kinase reaction velocity in the forward direction:

$$\text{creatine phosphate} \xrightarrow{k_{\text{forward}}} \gamma\text{-P of ATP}$$

Theoretical considerations underlying the technique have been presented previously (Forsen & Hoffman, 1963). In this study, two magnetization transfer pulse sequences were used, which we label Selsat and MoMz. In Selsat, the ATP γ-P peak was selectively irradiated with a low-power pulse (0.5 W) of increasing duration: 0.3, 0.6, 1.2, 2.4, and 4.8 s. The interpulse delay increased from 2.15 to 6.95 s during this sequence. Control spectra, without saturation, were obtained before and after this sequence. The relationship between magnetization M_{CP} and time of saturation was fitted to

$$f(x) = A \exp(-x/\tau) + B$$

where $A + B$ is M_0 (the area of the creatine phosphate resonance in the absence of saturation), B is M_∞ (the area of the creatine phosphate resonance at infinite saturation), and τ is magnetic lifetime. By use of

$$T_1 = M_0\tau/M_\infty$$

$$k = 1/\tau - 1/T_1$$

$$\text{reaction velocity} = kM_0$$

the T_1 for creatine phosphate, the pseudo-first-order rate constant for the forward creatine kinase reaction (k), and the reaction velocity of the forward creatine kinase reaction were calculated.

In the pulse sequence we label MoMz, two spectra were obtained by signal-averaging alternating scans. The first spectrum was obtained in the absence of saturation (M_0). For the second spectrum, ATP γ-P was selectively saturated for 5 s, approximating M_∞. For both spectra, the interpulse delay was 7.15 s. In this case, τ, k, and the reaction velocity were calculated from the T_1 obtained from the Selsat experiments.

Biochemical Analysis. Biochemical analyses were performed on all hearts used for NMR experiments, a group of hearts used for oxygen consumption measurements, and a group of nonperfused hearts. Following freeze-clamping, 5–15 mg of tissue was homogenized in potassium phosphate buffer, and aliquots were taken for the following measurements. Total creatine kinase activity was measured with the coupled enzyme scheme of Rosalki (1967) at 30 °C by use of the Calbiochem-Behring CK-NAC SVR kit. The ratio of activities at 37 to 30 °C was empirically determined to be 1.8. Distributions of the creatine kinase isoenzymes were determined by cellulose–acetate strip electrophoresis coupled with scanning fluorometry (Hall & DeLuca, 1967); results are expressed as percentage of total creatine kinase activity. Citrate synthase activity was measured according to the method of Srere (Srere et al., 1963). Measurement of total creatine was performed by the fluorometric assay of Kammermeier (1973). Enzyme activities are expressed as International Units (IU) per milligram of cardiac protein, measured according to the method of Lowry et al. (1951).

In a separate group of freeze-clamped hearts, ATP concentration was determined by high-pressure liquid chromatography for comparison with concentrations obtained by ^{31}P NMR. Briefly, frozen tissue was powdered in a stainless steel percussion mortar cooled in liquid nitrogen. The powder was homogenized in 0.4 N perchloric acid at 0 °C, and aliquots of the homogenate were removed for protein determination. The homogenate was neutralized and centrifugated for 5 min. The supernatant was used for the determination of ATP by applying aliquots to a Partasil SAX column. Nucleotides were eluted isocratically in 0.16 M K_2HPO_4 + 0.1 M KCl, pH 6.5, at room temperature at a flow rate of 1.4 mL/min (λ = 254 nm). Protein was measured by the method of Lowry et al. (1951). Results are expressed as nanomoles per gram dry weight by use of values for the ratios of grams of protein to grams wet weight and grams wet weight to grams dry weight.

Statistical Analysis. Fitting of the Selsat data was performed by variance-weighted nonlinear regression. Pairs of data were compared by the Student's t test. Multiple comparisons were made by the multiple range test of Newman–Keuls if a significant effect was found by one-way or two-way analysis of variance. Analysis of covariance was used to compare lines or curves. Values of $p < 0.05$ were considered significant. The above calculations, as well as regression analysis, were performed with the Research and Statistics Management Package (Bolt, Beranek and Newman, Cambridge, MA; VAX 11/780 Computer, Maynard, MA). Results, unless otherwise stated, are expressed as the means ± standard deviation.

Results

Three and 18 days postpartum (groups I and II, respectively) neonatal rabbits weighed 58 ± 13 and 261 ± 75 g and hearts weighed 380 ± 43 and 1370 ± 322 mg. Thus, heart and body weights increased approximately 4-fold between 3 and 18 days of age while the heart to body weight ratio did not change. Heart and body weights for animals used for oxygen consumption measurements and NMR experiments did not differ.

Biochemical Analyses. Results of creatine, protein, and enzyme analyses are displayed in Table I. Because total creatine kinase, expressed either as IU (mg of protein)$^{-1}$ or IU (mg of tissue)$^{-1}$, did not change during the period of development studied, expressing the creatine kinase isoenzymes as a percentage of total creatine kinase activity reflects the relative tissue activities of the isoenzymes. During the period of development studied, total creatine content and the percentage of total creatine kinase activity present as MM-CK and mito-CK increased, while the percentages of BB-CK and MB-CK decreased. Dividing group II hearts into two groups on the basis of percentage of mito-CK (≤6% or >6%) yielded three groups of hearts with different mito-CK activities: group I (0% mito-CK), group IIa (3.7% mito-CK), and group IIb (9.3% mito-CK). Although citrate synthase activity, a marker of mitochondrial protein, increased slightly between 3 and 18 days of age, the change was not significant. When group IIa and IIb hearts were compared, the only difference in any of these parameters was in mito-CK activity.

The ratio of activities of mito-CK (calculated by multiplying mito-CK, expressed as the percentage of total creatine kinase, by total creatine kinase activity) to citrate synthase increased from 0 in group I to 0.73 in group IIa and 1.66 in group IIb, demonstrating not only an absolute increase in the tissue content of mito-CK but also an increase relative to mitochondrial protein.

Measurement of ATP and creatine phosphate concentrations in hearts by standard biochemical methods yielded results that did not differ significantly from those obtained by NMR.

There were no differences in any of these parameters between perfused and nonperfused hearts nor between hearts

Table I: Biochemical Analysis of Hearts[a]

	protein [mg (mg ww)$^{-1}$]	IU (mg of protein)$^{-1}$		% distribution				creatine [μmol (g dry weight)$^{-1}$]
		CS	CK	BB	MB	MM	mito	
group I (3 days old)								
mean	0.13	0.37	8.73	3.1	22.4	74.5	0	16.7
SD	0.02	0.07	1.11	1.6	5.9	4.8		2.8
group II (18 days old)								
mean	0.16	0.47	9.02	1.2	7.5	85.8	5.5	54.0
SD	0.05	0.15	2.06	1.4	2.9	5.7	3.3	15.1
p	0.05	0.001	NS	0.001	0.001	0.01	0.001	0.001
group IIa (18 days old, <6% mito-CK)								
mean	0.16	0.49	9.30	1.3	7.4	87.6	3.7	53.5
SD	0.05	0.16	2.14	1.3	2.8	4.2	1.8	16.2
group IIb (18 days old, >6% mito-CK)								
mean	0.18	0.45	8.61	1.2	7.6	81.9	9.3	61.0
SD	0.05	0.13	1.57	1.4	3.0	5.4	2.7	16.2
p	NS	NS	NS	NS	NS	NS	0.001	NS

[a] Hearts used for ^{31}P NMR were frozen and analyzed to determine protein [mg (mg wet weight)$^{-1}$] and creatine [μmol (g dry weight)$^{-1}$] contents, citrate synthase (CS) and total creatine kinase (CK) activities [IU (mg of protein)$^{-1}$], and the distribution of the creatine kinase isoenzymes, BB-CK, MB-CK, MM-CK and mito-CK (percentage of total creatine kinase activity). Values represent the mean ± standard deviation (SD) of 5–10 hearts. Included are p values for comparisons between groups I and II and between groups IIa and IIb, where $p > 0.05$ was not significant (NS).

Table II: Relationship between Oxygen Consumption and Cardiac Performance[a]

	oxygen consumption			10^{-3} × RPP, isovolumic beating hearts
	KCl arrest	Langendorff	isovolumic, beating hearts	
3 days old	n = 18	n = 10	n = 21	
	4.6 ± 0.7	12.2 ± 1.5	17.6 ± 2.4	15.1 ± 2.2
18 days old	n = 20	n = 20	n = 45	
	4.2 ± 1.1	11.7 ± 2.0	17.5 ± 3.1	16.1 ± 2.9

[a] Oxygen consumption [μmoles of O_2 (g dry weight)$^{-1}$ min^{-1}] was measured in hearts from groups I (3 days old) and II (18 days old) at three levels of cardiac performance: KCl-arrested hearts; empty, beating hearts (Langendorff); isovolumic, beating hearts. For isovolumic, beating hearts, performance is expressed as rate–pressure product (RPP; heart rate multiplied by developed pressure, mmHg min^{-1}). Values are mean ± standard deviation and do not differ significantly between groups.

used for NMR and oxygen consumption measurements.

Oxygen Consumption Measurements. For any level of cardiac performance, values for oxygen consumption (Table II) were indistinguishable between 3-day-old and 18-day-old hearts and between groups of 18-day-old hearts (groups IIa and IIb). The regression equations relating oxygen consumption and rate-pressure products were $Y = 0.153X + 0.91$ ($r = 0.95$) (group I) and $Y = 0.149X + 0.82$ ($r = 0.94$) (group II), where Y is oxygen consumption in μmol of O_2 (g wet weight)$^{-1}$ min^{-1} and X is 10^{-3} RPP mmHg min^{-1}. For both groups, oxygen consumption increased approximately 4-fold as cardiac performance increased from low levels in the KCl-arrested hearts to high levels in the isovolumic, beating hearts. The regression equations were used to estimate oxygen consumption in isovolumic, beating hearts from the NMR experiments.

^{31}P NMR Experiments. ATP and creatine phosphate concentrations (Table III) and intracellular pH were determined for each group of hearts by ^{31}P NMR spectroscopy (examples of spectra are shown in Figure 1). The ATP concentration did not change during development nor with changes in cardiac performance and was 20.5 ± 3.8 μmol (g dry weight)$^{-1}$ in 3-day-old hearts and 21.8 ± 3.9 μmol (g dry weight)$^{-1}$ in 18-day-old hearts. The creatine phosphate concentration increased 3-fold, from 6.52 ± 2.1 to 19.5 ± 5.8 μmol (g dry weight)$^{-1}$, over the age range studied. The intracellular

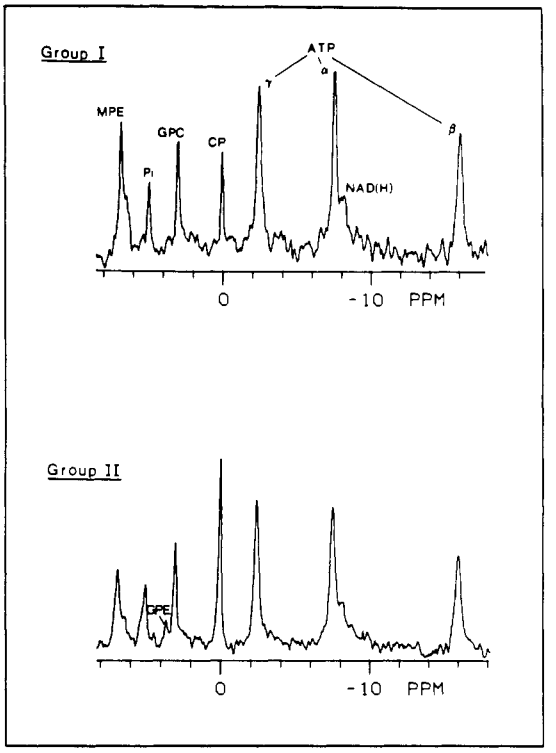

FIGURE 1: ^{31}P NMR spectra from a group I heart with no mito-CK performing at an RPP of 17 500 mmHg min^{-1} and a group II heart with 7.2% mito-CK performing at an RPP of 18 000 mmHg min^{-1}. Peaks representing the monophosphate esters (MPE), inorganic phosphate (P_i), *sn*-glycerophosphoethanolamine (GPE), *sn*-glycerophosphocholine (GPC), creatine phosphate (CP), the α, γ, and β peaks of ATP, and NAD(H). The spectra demonstrate the increase in the creatine phosphate to ATP ratio which occurs during development.

pH averaged 7.1 and did not differ among groups or change with cardiac performance.

Magnetization transfer provided measurements of the spin–lattice nuclear relaxation parameter, T_1 (measured in the presence of saturation but in the absence of exchange), the pseudo-first-order rate constant, and the reaction velocity for the transfer of high-energy phosphate from creatine phosphate to ADP to form ATP. The T_1 for creatine phosphate, averaging 2.1 ± 0.2 s, did not differ among groups or change with cardiac performance. When matched by group and level of

Table III: Metabolite Concentrations (mM) for the Creatine Kinase Reaction[a]

	creatine	CP	ATP	ADP
KCl arrest				
group I (0% mito-CK)	3.5 ± 0.9	2.5 ± 0.09	7.8 ± 1.6	0.07 ± 0.04
group IIa (3.7% mito-CK)	11.7 ± 5.4	7.2 ± 1.0	7.9 ± 1.4	0.08 ± 0.04
group IIb (9.3% mito-CK)	12.4 ± 3.7	8.6 ± 2.2	8.2 ± 1.6	0.07 ± 0.03
isovolumic, beating				
group I (0% mito-CK)	3.6 ± 0.5	2.5 ± 0.5	7.5 ± 1.5	0.07 ± 0.02
group IIa (3.7% mito-CK)	12.1 ± 5.6	6.3 ± 1.9	8.1 ± 1.1	0.10 ± 0.07
group IIb (9.3% mito-CK)	14.9 ± 5.1	7.5 ± 1.6	8.2 ± 1.6	0.10 ± 0.03

[a] The free creatine concentrations were obtained by subtracting the creatine phosphate concentration (CP) from the total creatine concentration. Creatine phosphate and ATP concentrations were measured by ^{31}P NMR, and ADP concentrations were estimated from an equilibrium constant of 166 for the creatine kinase reaction. Concentrations, expressed as mM, were converted from μmol (g wet weight)$^{-1}$ with 2 g wet weight (g of intracellular water)$^{-1}$. Mean values ± standard deviation are shown.

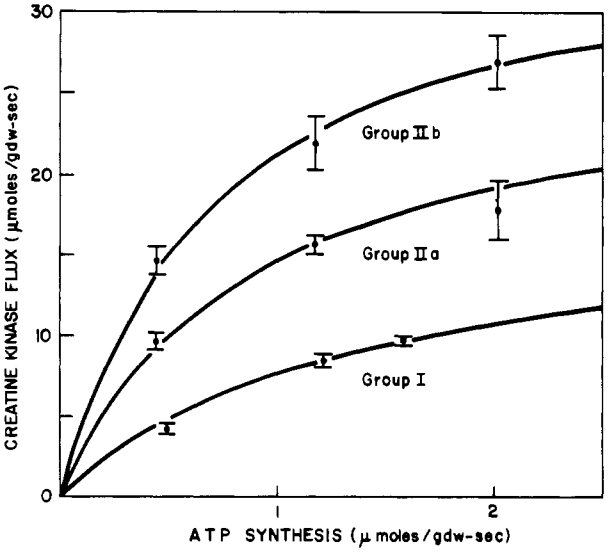

FIGURE 2: Relationship between the unidirectional creatine kinase reaction velocity (flux) [μmol (g dry weight)$^{-1}$ s^{-1}] measured by magnetization transfer and rate of ATP synthesis [μmol (g dry weight)$^{-1}$ s^{-1}] calculated from oxygen consumption measurements assuming a P:O ratio of 3. The curves are the result of fitting all the data points (not the mean values which are shown) for each group to the Michaelis–Menten equation (see text). The three points shown for each group or curve represent means for each group at the three levels of cardiac performance. Error bars represent standard error.

cardiac performance, there were no differences in rate constant or in the measured reaction velocity derived from data obtained by Selsat or MoMz, and therefore, the data from the two pulse sequences were combined for analysis.

Table IV shows values for the pseudo-first-order rate constants, k, and the reaction velocities for the three groups at each of the three levels of performance studied. Analyzing the data by row (i.e., at constant mito-CK activity) shows that, for each group, the creatine kinase reaction velocity was ~ 2-fold higher in beating hearts than in arrested hearts. Thus, the observation that the reaction velocity is higher in beating hearts than in nonbeating hearts is independent of creatine kinase isoenzyme distribution. Analyzing the data by column (i.e., at constant level of cardiac performance) shows that the reaction velocity increased from group I to group IIa to group IIb and the total increase was nearly 3-fold. Thus, as mito-CK activity increased, the creatine kinase reaction velocity increased.

Table V shows the ratios of the creatine kinase reaction velocity to the rate of ATP synthesis estimated by use of oxygen consumption for each group at each level of cardiac performance. For each of the nine conditions studied, creatine kinase reaction velocity was an order of magnitude faster than net rate of ATP synthesis. Analyzing the data by row permits comparison of the ratio for each group of hearts as workload increased at constant mito-CK activity: the ratio decreased as workload increased. This suggests that the relative proportion of ATP synthesis via creatine kinase decreases as ATP synthesis rate via oxidative phosphorylation increases. The ratio changed the least (~50%) in hearts with no mito-CK activity and the most in hearts with high mito-CK activity (~160%). Analyzing the data by column permits comparison of the ratio as the mito-CK activity increased, but workload was constant. This comparison shows that, at constant workload, the ratio increased as mito-CK activity increased. For arrested hearts, the ratio increased ~4-fold. For isovolumic hearts, the ratio increased only 2-fold. Thus, compared to the rate of ATP synthesis via oxidative phosphorylation, the rate of ATP synthesis catalyzed by creatine kinase is highest in hearts with the highest mito-CK activity operating at low workloads and lowest in hearts with the lowest mito-CK activity operating at high workloads.

Table IV: Magnetization Transfer Results for the Creatine Kinase Reaction[a]

	Group I (0% Mito-CK)									
	KCl arrest (n = 5)			Langendorff (n = 6)			isovolumic, beating heart (n = 10)			
	CP	k	V	CP	k	V	CP	k	V	10^{-3} × RPP
mean	7.1	0.67	4.3	6.3	1.53	8.6	5.8	1.57	9.5	13.3
SD	2.7	0.25	0.6	2.5	0.53	1.0	0.9	0.31	0.7	3.3
	Group IIa (3.7% Mito-CK)									
	KCl arrest (n = 8)			Langendorff (n = 6)			isovolumic, beating heart (n = 8)			
	CP	k	V	CP	k	V	CP	k	V	10^{-3} × RPP
mean	19.3	0.51	9.7	15.3	1.09	15.7	17.5	1.06	17.9	19.6
SD	2.4	0.01	1.4	4.3	0.29	1.53	5.8	0.26	5.2	4.6
	Group IIb (9.3% Mito-CK)									
	KCl arrest (n = 6)			Langendorff (n = 4)			isovolumic, beating heart (n = 9)			
	CP	k	V	CP	k	V	CP	k	V	10^{-3} × RPP
mean	21.8	0.68	14.7	29.5	0.80	22.0	18.4	1.48	27.1	19.5
SD	3.1	0.10	2.1	9.6	0.23	3.3	2.6	0.24	5.1	2.4

[a] Creatine phosphate concentration [CP; μmol (g dry weight)$^{-1}$], rate constants for the forward creatine kinase reaction (k; s^{-1}), and velocity (V) of the forward creatine kinase reaction [μmol (g dry weight)$^{-1}$ s^{-1}] obtained by ^{31}P NMR. Mean values ± standard deviation for groups I, IIa, and IIb at three levels of cardiac performance (RPP; rate–pressure product, mmHg min^{-1}) are shown.

Table V: Ratio of Creatine Kinase Reaction Velocity to Rate of ATP Synthesis[a]

	reaction velocity/ATP synthesis		
	KCl arrest	Langendorff	isovolumic, beating
group I (0% mito-CK)	9.3	7.0	6.0
group IIa (3.7% mito-CK)	23.0	13.4	8.9
group IIb (9.3% mito-CK)	35.0	18.8	13.5

[a] The ratios of the unidirectional reaction velocity [μmol (g dry weight)$^{-1}$ s^{-1}] measured by ^{31}P NMR magnetization transfer to the rate of ATP synthesis [μmol (g dry weight)$^{-1}$ s^{-1}] estimated from oxygen consumption and assuming a P:O ratio of 3 are shown for groups I, IIa, and IIb at three levels of cardiac performance. Values represent the quotient of means.

In Figure 2, the creatine kinase reaction velocity is plotted against the rate of ATP synthesis estimated from oxygen consumption measurements for groups I, IIa, and IIb. While the three points on each curve represent the mean ± standard error for arrested and Langendorff and isovolumic beating hearts, the curves were obtained by fitting all the data points for each group (not the three mean values shown) to an equation with the form

$$V = V_{max}\frac{ATPs}{ATPs + ATPs50}$$

where V is the unidirectional reaction velocity, V_{max} is the maximum reaction velocity, ATPs is the rate of ATP synthesis (from oxygen consumption) and ATPs50 is the rate of ATP synthesis at which the creatine kinase reaction velocity is half-maximal (Bittl & Ingwall, 1985). With this equation, the fits had r values of 0.99, 0.99, and 0.97 for groups I, IIa, and IIb, respectively. For comparison, use of linear regression yielded r values of 0.78, 0.80, and 0.66. ATPs50 decreased from 1.40 to 0.90 to 0.69 μmol (g dry weight)$^{-1}$ s^{-1}, and V_{max} increased from 18 to 28 to 36 μmol (g dry weight)$^{-1}$ s^{-1} in groups I, IIA, and IIB, respectively.

When the data for body weight, heart weight, creatine phosphate concentration, oxygen consumption, percentage of mito-CK of total creatine kinase activity, and the creatine kinase reaction velocity from all of the hearts were analyzed by multiple regression analysis with reaction velocity as the dependent variable, the following parameters were found to contribute to the regression: oxygen consumption (p = 0.0001), percentage mito-CK (p = 0.0021), creatine phosphate concentration (p = 0.004), and heart weight (p = 0.013). When the data from only groups IIa and IIb were analyzed, level of cardiac performance (p = 0.0001), percentage of mito-CK of total creatine kinase activity (p = 0.001), and creatine phosphate concentration (p = 0.042) contributed to the regression. Figure 3 shows the relationship between the reaction velocity and percentage mito-CK for all three groups of hearts.

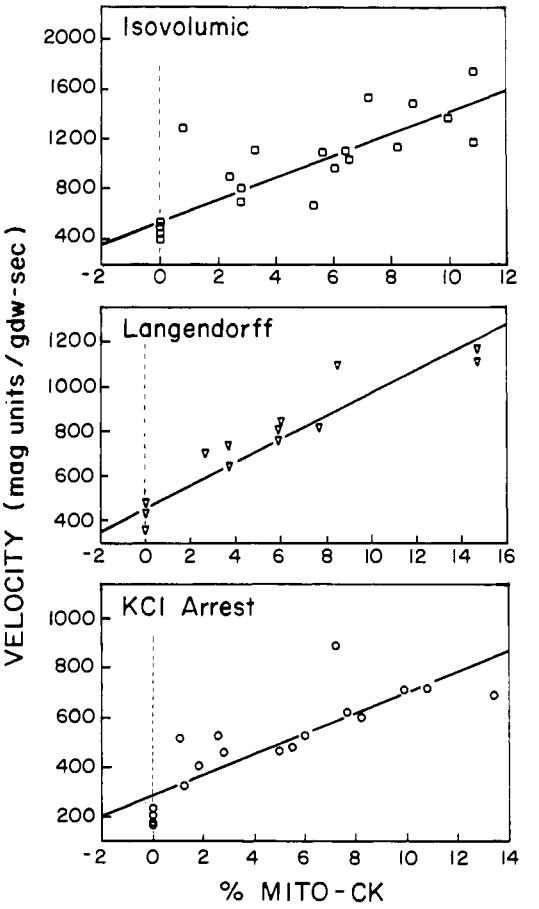

FIGURE 3: Relationship between the unidirectional creatine kinase reaction velocity (velocity) [magnetization units (g dry weight)$^{-1}$ s^{-1}] and mito-CK (percent of total creatine kinase) for KCl-arrested and Langendorff and isovolumic beating hearts from groups I (0% mito-CK), IIa (3.7% mito-CK), and IIb (9.3% mito-CK). r^2 values are 0.731 for isovolumic, 0.905 for Langendorff, and 0.727 for KCl-arrested hearts.

Discussion

In this study, the velocity of the creatine kinase reaction, measured by ^{31}P magnetization transfer, was studied in isolated, perfused hearts at different levels of cardiac performance and from different stages of development. Under the steady-state conditions studied, the forward creatine kinase reaction velocity (ATP production) equals the reverse creatine kinase reaction velocity (creatine phosphate production), and ATP synthesis rates, estimated from oxygen consumption measurements, equal ATP utilization rates. The results demonstrate that the creatine kinase reaction velocity changes with the size of the creatine pool, the creatine kinase isoenzyme distribution, and the rate of ATP synthesis. These relationships will now be considered in detail.

Substrate Regulation. The concentrations of each of the substrates in the creatine kinase reaction, ATP, creatine phosphate, ADP, and creatine, were measured or (for ADP) estimated. The measured concentrations are averages, both with respect to localization within the cell and with respect to the cardiac cycle. The ATP concentration [21 μmol (g dry weight)$^{-1}$] obtained in this study is similar to reported values for immature rabbit myocardium (Jamarkani et al., 1978a,b) and did not change over the range of development or cardiac performance studied. However, the creatine phosphate concentration in 3-day-old hearts (group I) [6 μmol (g dry weight)$^{-1}$] obtained by both standard biochemical techniques and NMR in the present study is lower than previously reported values [22 μmol (g dry weight)$^{-1}$] (Jamarkani et al., 1978a,b). In fact, the creatine phosphate concentrations reported by Jamarkani et al. (1978a,b) are higher than the total creatine concentration found in 3-day-old hearts in our study. We found that creatine and creatine phosphate contents increased 3-fold during the period of development studied. This is similar to age-related increases observed in other species (Ingwall et al., 1980). The concentration of unbound ADP, which cannot be measured directly, was estimated with an equilibrium constant for the creatine kinase reaction of 166 (Veech et al., 1979) and varied between 60 and 100 μM. These values are higher than those reported for the mature rat heart and reflect the higher free creatine to creatine

Table VI: Velocity of the Creatine Kinase Reaction: Predicted versus Measured[a]

		velocity (mM s^{-1})		% increase (arrest to isovolumic)
		arrest	isovolumic	
group I (0% mito-CK)	predicted	8.6	8.5	−1
	measured	1.7	3.3	94
group IIa (3.7% mito-CK)	predicted	20.2	21.7	8
	measured	3.6	6.9	92
group IIb (9.3% mito-CK)	predicted	20.2	23.5	16
	measured	5.5	10.1	84
% increase comparing				
I to IIa	predicted	135	155	
	measured	112	109	
IIa to IIb	predicted	0	8	
	measured	53	46	

[a] Values for the forward reaction velocity (mM s^{-1}): $V(\text{for}) = (V_{max}(\text{for})[\text{ADP}][\text{CrP}])/[DK_m(\text{ADP})K_i(\text{CrP})]$, where $D = 1 + [\text{ADP}]/K_i(\text{ADP}) + [\text{CrP}]/K_i(\text{CrP}) + [\text{ATP}]/K_i(\text{ATP}) + [\text{Cr}]/K_i(\text{Cr}) + ([\text{ADP}][\text{CrP}])/[K_m(\text{ADP})K_i(\text{CrP})] + ([\text{ATP}][\text{Cr}])/[K_m(\text{ATP})K_i(\text{Cr})] + ([\text{ADP}][\text{Cr}])/[K_m(\text{ADP})K_i(\text{Cr})]$. These were calculated with the creatine kinase rate equation (Morrison & Cleland, 1966) or measured by NMR. Substrate concentrations for the calculations are from Table III, and K_m (mM) and K_i (mM) values are averages taken from Bittl and Ingwall (1987). K_m of MM-CK and MB-CK: MgADP, 0.08; CrP, 2.1; MgATP, 1.2; Cr, 21.3. K_m of mito-CK: MgADP, 0.05; CrP, 2.0; MgATP, 0.93; Cr, 17.3. K_i of MM-CK: MgADP, 0.09; CrP, 1.04; MgATP, 0.9; Cr, 20. K_i of mito-CK: CrP, 1.4; MgATP, 0.29; Cr, 29.4.

phosphate ratio observed for the neonatal rabbit heart (1.85 ± 0.26) compared with the mature rat heart (0.7–1.1) (Bittl & Ingwall, 1985).

To assess the contribution of changes in average cytosolic substrate concentrations in regulating the reaction velocity, we calculated the creatine kinase reaction velocity using the rate equation developed by Morrison and Cleland (1966) and amended by Kupriyanov et al. (1984). The K_m and K_i values for each substrate and isoenzyme represent averages from the literature (none from rabbits) (Bittl et al., 1987a,b) weighted for the isoenzyme distribution of each group of hearts. In contrast to results for rat heart in situ or in vitro (Bittl & Ingwall, 1987), the correlation between velocities predicted by the rate equation and velocities measured by magnetization transfer is poor. This could be due to use of inappropriate values for K_m or K_i. It is also possible that the rate equation as formulated is incomplete. If the poor correlation is due to inappropriate K_m and/or K_i values, then relative changes in predicted and measured reaction velocities should be the same. In Table VI, predicted and measured reaction velocities for each group of hearts for two levels of cardiac performance, arrest and beating, are given. Also shown are values for the percentage difference in predicted and measured reaction velocities obtained by comparing values for beating versus arrested hearts and by comparing differences among groups at each level of cardiac performance. When the creatine pools increased 3-fold (compare groups I and IIa at each workload), both predicted and measured reaction velocities increased 110–150%. However, the rate equation fails to predict measured changes in the reaction velocity associated with changes in the rate of ATP synthesis (compare different levels of cardiac performance for each group) and associated with changes in mito-CK activity (compare groups IIa and IIb at similar levels of cardiac performance). Thus, relative changes in predicted and measured reaction velocities are similar only when there are large changes in average cytosolic substrate concentrations.

Role of Isoenzyme Distribution. During the period of development studied, total creatine kinase activity is constant and cannot explain observed changes in the reaction velocity. However, there are significant changes in the creatine kinase isoenzyme distribution. Between 3 and 18 days of age (groups I and II), mito-CK activity appears and accumulates to ~6% of the total, MM-CK activity increases from 75 to 86%, and the combined BB-CK and MB-CK activities decrease from 25 to 9% of total creatine kinase activity. These changes are qualitatively similar to developmental changes in other species (Ingwall et al., 1980). Hearts from 18-day-old rabbits (group II) were divided into two groups on the basis of the amount of mito-CK activity present, yielding three groups of hearts with different mito-CK activities: group I with 0% (3 day old), group IIa with 3.7% (range 1–6%), and group IIb with 9.3% (range 6–13%) of total creatine kinase activity present as mito-CK. Adult rabbit hearts contain ~15% mito-CK activity.

The relationship between changes in creatine kinase isoenzyme distribution, specifically in mito-CK activity, and the reaction velocity is demonstrated by comparing groups IIa and IIb at similar rates of ATP synthesis. The only significant differences between these groups are mito-CK activity (3.7% versus 9.3%) and the 1.5-fold increase in the creatine kinase reaction velocity. A causal relationship between creatine kinase isoenzyme distribution and regulation of the reaction velocity is suggested by analysis of the data with an equation of the Michaelis–Menton form (Bittl & Ingwall, 1985). That analysis (Figure 2) suggests that as mito-CK activity increases the rate of ATP synthesis at which the reaction velocity is half-maximal (ATPs50) decreases and the maximal reaction velocity (V_{max}) increases. Given the similarity in kinetic properties of the isolated isoenzymes, simply substituting one isoenzyme for another would not be expected to alter the relationship between the rate of ATP synthesis and the creatine kinase reaction velocity. Instead, our results are consistent with experiments using isolated mitochondria in which the apparent K_m of mito-CK for ATP was 5-fold lower when mito-CK was bound to the mitochondria than when it was unbound (Saks et al., 1980), demonstrating the importance of localization of this isoenzyme. Our results also show that as mito-CK activity increased V_{max} increased despite no change in total creatine kinase activity. In contrast, with isolated mitochondria (Saks et al., 1980), V_{max} was the same for bound and unbound mito-CK. This suggests that, in the intact cell, the effective creatine kinase activity depends on the distribution and, therefore, localization of the isoenzymes (mito-CK on the mitochondria and MM-CK at sites of ATP utilization). On the basis of this analysis, we postulate that, due to isoenzyme localization (as well as the presence of competing reactions), the number of active sites for conversion of ATP produced by oxidative phosphorylation to creatine phosphate is effectively greater when mito-CK is bound to mitochondria than when only cytosolic creatine kinase is present.

Relationship between ATP Synthesis via the Creatine Kinase Reaction and by Oxidative Phosphorylation. The relationship between changes in the rate of ATP synthesis (estimated for oxygen consumption measurements by using a P:O ratio of 3) and the creatine kinase reaction velocity remains controversial. Studies of the arrested rat heart (Bittl & Ingwall, 1986) and those comparing arrested and beating rat hearts (Bittl & Ingwall, 1985; Kupriyanov et al., 1984) have found the creatine kinase reaction velocity increases as oxygen consumption increases. Other studies (Matthews et al., 1982; Shoubridge et al., 1985) comparing narrower ranges of cardiac performance in the beating rat heart have not detected changes in the reaction velocity. Our results comparing arrested and isovolumic, beating hearts for all three groups of rabbit hearts

demonstrate that the reaction velocity increases 2-fold as oxygen consumption increases 4-fold. This is similar to results obtained in mature rat hearts (Bittl & Ingwall, 1985). However, when data are compared for only *beating* rabbit hearts (Langendorff versus isovolumic beating hearts) at each stage of development, a different result is obtained. For hearts with less than 6% mito-CK activity (groups I and IIa), there was no significant change in the creatine kinase reaction velocity as ATP synthesis via oxidative phosphorylation increased. We observed an increase in reaction velocity as oxygen consumption increased only in beating hearts with high mito-CK activity (group IIb). As reported (Bittl & Ingwall, 1985) and confirmed here, the relationship between oxygen consumption (ATP synthesis rate) and the reaction velocity is best described by a hyperbola. As a result, at higher levels of oxygen consumption, where the curve flattens, large changes in oxygen consumption are needed to produce changes in the creatine kinase reaction velocity. Thus, our observations suggest that increases in creatine kinase reaction velocity with oxygen consumption are observed only when a relatively high proportion of total creatine kinase activity is localized on the mitochondria. Consistent with this hypothesis, creatine kinase reaction velocity did not increase with stimulation in skeletal muscle of the hindlimb of the rat, which contains only 2% mito-CK (Zahler et al., 1987). Our observations may also explain the results of Malloy and colleagues (MacDonald et al., 1986), showing no increase in creatine kinase reaction velocity as workload increased in the beating dog heart, which contains approximately 9% mito-CK (Ingwall et al., 1984).

In summary, this study using the developing rabbit heart defines the relationships among creatine kinase reaction velocity, creatine kinase isoenzyme distribution, size of the creatine pool, and ATP synthesis rates by oxidative phosphorylation. We have found that, first, at a constant rate of ATP synthesis, both the creatine kinase reaction velocity and V_{max}, the maximal reaction velocity, increased as mito-CK activity increased. Second, expanding the creatine pool increased the reaction velocity. Third, the reaction velocity increased as the rate of ATP synthesis increased, regardless of isoenzyme distribution and despite only small changes in average cytosolic substrate concentrations.

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