Kinetics of creatine kinase in an experimental model of low phosphocreatine and ATP in the normoxic heart


Abstract: To study the dependence of the forward flux of creatine kinase (CK) on its substrates and products, we designed an acute normoxic model of steady-state depletion of phosphocreatine (PCr) and adenylyl in the isovolumic acute desighed an acute normoxic model of steady-state depletion of creatine kinase (CK) on its substrates and products we

Equilibrium at all points of the cell. This implies that the concentrations of substrates and products of the reaction are identical at all intracellular localization because of facilitated diffusion. A corollary of this approach is that, in vivo, CK flux must be governed by the concentrations of ATP, PCr, Cr, ADP, and H⁺. Another approach to CK function is based on the existence of CK compartmentation by the localization of CK isozymes close to sites of energy production and utilization in the cell. MM-CK isozyme bound in the vicinity of adenosinetriphosphatases (ATPases) affects their apparent kinetics (15) and translocase at the site of mitochondrial energy production (19), which has led to the concept of energy channeling by a PCr-Cr-CK system in the myocardium (19).

In vitro, the known kinetics of CK in dilute solution (11) are adequately described by NMR magnetization-transfer methods (8). In vivo CK kinetics have also been extensively studied by NMR. Three main factors have been proposed to govern the CK flux in vivo: total CK activity, CK isozymic composition, and concentration of products and substrates. The importance of isozymic composition was first pointed out during neonatal development; at constant total CK activity, CK flux increased together with mito-CK content (18). In vivo CK velocity on substrate concentration has been analyzed by comparing various species during neonatal development (13, 18) or by changing the size of the guanidine pool by long-term feeding with Cr analogs (22). However, in all these cases several parameters are affected besides the metabolite concentrations. For example, during perinatal development, in addition to an increase in guanidine pool, a complete reorganization of the cell occurs, as evidenced by the expression of the mito-CK isozyme, the presence of functional coupling of both MM-CK to ATPase and mito-CK to translocase, and the increase in energetic requirements (6).
plasticity of the cardiac muscle, could the specific contribution of change in substrate and product concentrations to CK velocity easily be separated from cellular remodeling.

Our aim, therefore, was to design an acute experimental model to study the effects of large changes of CK metabolite concentrations per se on CK velocity. We took advantage of the model of adenylate depletion (based on intracellular trapping of phosphorus that we previously developed; Ref. 7), to design a new experimental model of steady-state heart contractility and metabolite concentrations with large reductions in both PCr and adenylate concentrations. We measured the apparent rate constant (k₂) and the flux of CK in the forward direction (PCr→ATP) by NMR progressive saturation transfer for a threefold change in PCr content and a sevenfold decrease in ATP. Up to now there has been to our knowledge no analysis of myocardial CK velocity under such a wide change of both guanidylate and adenylate concentrations. In this nonglycolytic model, contractility is sustained as previously described. Here we show experimental evidence of sustained cytosolic transfer of energy at high ATP content. The CK forward flux was unchanged despite a threefold decrease in PCr content due to an increase in k₂. The independence of CK flux with regard to PCr concentration is indeed observed in dilute solution in vitro because of the equilibrium behavior of CK. However, at variance with the concept of equilibrium, CK flux decreased at low ATP concentration. The reasons for such discrepancy are tentatively discussed in terms of the relative contribution of the CK isoforms to the NMR-measured total CK flux.

MATERIALS AND METHODS

Physiology

Animal experimentation was performed in accordance with the American Heart Association’s position statement on research animal usage. Wistar male rats (350–450 g) were anesthetized with ethyl carbamate (2g/kg), and hearts were perfused by the Langendorff technique at a constant flow of 13.5 ml/min. The left ventricle (LV) was pierced to avoid fluid accumulation. A latex balloon was inserted in the LV and inflated with 2H₂O to isovolumic conditions of work. LV accumulation. A latex balloon was inserted in the LV and inflated with 2H₂O to isovolumic conditions of work. LV pressure and coronary pressure were recorded with Statham gauges and continuously monitored on a computer (Compaq). The perfusion solution included 10 additional hearts in which PCr content was maintained at various levels ranging from 90 to 30% of control PCr content when this steady state was achieved. The 2DG-1 group also included four protocols: a control group (n = 6) and three groups with various PCr concentrations ([PCr]) achieved by 2DG perfusion: protocol 2DG-1, with intermediate ATP concentration ([ATP]; i.e., >50%) and pH₇.₃₅ (n = 18), and protocols 2DG-2 and 2DG-2a, with low [ATP] (i.e., <50%) and pH₇.₃₅ (n = 18) and 7.₆₅ (n = 5), respectively.

In protocol 2DG-1 (variable [PCr] at medium [ATP]), eight hearts were perfused with 1 mM 2DG in the presence of 4 IU/L insulin until PCr was decreased to 50% as checked on the spectrum; the duration of 1 mM 2DG perfusion was 10.₆ ± 1.₉ min. On removal of the 2DG + insulin solution, 0.075 mM 2DG without insulin was continuously infused to prevent PCr recovery. Saturation transfer was performed when this steady state was achieved. The 2DG-1 group also included 10 additional hearts in which PCr content was adjusted to various levels ranging from 90 to 30% of control PCr value by changing the duration (4–12 min) and the concentration (1–2 mM) of the initial 2DG perfusion. To maintain a steady state, the concentration of 2DG infusion was adjusted (0.₆₆–0.₈₈ mM) as a function of 2DG6P content.

Protocol 2DG-2 (variable [PCr] at low [ATP]) achieved the same range of PCr contents as in 2DG-1 but at low ATP content. Massive decrease in high-energy phosphates was induced in the presence of insulin by perfusion either for 19.₄ ± 0.₆ min in the presence of 2 mM 2DG (n = 12) or for 15.₀ ± 0.₅ min in the presence of 5 mM 2DG (n = 6). The two protocols were equivalent, and data were pooled. At this stage, the PCr peak had disappeared from the NMR spectra. In nine hearts, PCr was allowed to recover to 50% of its original value by removal of 2DG in the presence of insulin. As in the previous protocol, steady-state PCr content was main-
tained by infusion of 0.075 mM 2DG. In nine additional hearts, a variation in the duration of 2DG-free insulin perfusion allowed different levels of PCr recovery, from 30 to 75% of control. As in protocol 2DG-1, the concentration of 2DG in the infusion was adjusted for steady state. Protocols 2DG-1 and 2DG-2 induced intracellular acidification. To check its influence, we designed a 2DG-2 protocol, protocol 2DG-2a (same PCr and ATP as 2DG-2), in which intracellular pH (pH_i) was restored to control by external alkalinization. After a low ATP and a PCr content of 50% similar to 2DG-2 were reached, pH_i was increased from 7.35 to 7.65; as a consequence, pH_i recovered to its control value. In these hearts (n = 5), saturation transfer was performed at both pH_i with 24 scans in each spectrum.

NMR

31P NMR spectra were acquired at 161.93 MHz on a Bruker AM 400 wide-bore magnet in 20-mm-diameter tubes. Homogeneity was made on the heart water, and the frequency was locked on -H2O contained in the LV balloon. We used a pulse angle of 50° measured on the γ-ATP signal, acquisition of 4,000 data points, a spectral width of 10,000 Hz, and a line broadening of 20 Hz. The control period acquired after 10 min of equilibration in isovolumic conditions included four spectra of 64 scans with an interpulse delay of 2 s and one spectrum of 32 scans with 10-s interpulse delay for equilibrium values. The signals of 2DG6P, PCr, and β-ATP were reduced by saturation to 78, 66, and 96%, respectively, of their equilibrium value. The same NMR parameters (interpulse delay 2 s) were used to follow PCr depletion by 2DG and to check the steady state after depletion by comparing four spectra taken just before and just after saturation transfer was performed. Hearts with >10% change in PCr or ATP content before and after saturation were not included in the study.

The forward CK rate constant was measured by time-dependent saturation transfer (4) using the Dante method (16). The pulse angle was 1.6–1.8 µs, the delay between pulses was 5.5 ms, and the number of pulses ranged from 545 to 16,360. The durations of saturation were calculated to be equally distributed on the fitting curve. Each determination of CK rate constant included nine spectra, one nonsaturated spectrum with a rate of recurrence of 10 s allowing an of CK rate constant included nine spectra, one nonsaturated equally distributed on the fitting curve. Each determination to 16,360. The durations of saturation were calculated to be 5.5 ms, and the number of pulses ranged from 545 to 16,360. The delays between pulses were adjusted to achieve in each spectrum a constant rate of recurrence of 10 s. Free induction decays were acquired by trains of eight scans cycling five to six times through the protocol (total number of scans for each spectrum = 40–48). This procedure of signal averaging minimized the possible influence of any change in contractility or energetics that might occur during the saturation-transfer experiment. In six 2DG-1 hearts, the flux was measured in the same heart during control and after PCr depletion. In this case, 24 scans for each spectrum were used for determination of the control flux and the values were similar to the control group; all data were pooled.

Metabolite concentrations and NMR-measured forward flux of CK. Some hearts were freeze clamped at the end of the experiment, and biochemical analysis of PCA extracts was performed to measure ATP, PCr, and Cr content as previously described (7). Values were expressed in nanomoles per milligram of protein. Biochemical determination confirmed that none of the experimental conditions induced Cr leakage. Cr was thus calculated from the difference between total Cr plus PCr measured biochemically at the end of the experiment and PCr measured on each spectrum.

NMR quantification was performed with a home-made program on the area of each peak corrected for saturation. Biochemically determined PCr content of 43 nmol/mg protein for control hearts was used as an internal standard, and cytosolic volume was taken as 2.72 µl/mg protein. pHi was determined from the shift of Pi, or 2DG6P peak with respect to PCr. Free ADP was calculated from the equilibrium of the CK reaction with the apparent equilibrium constant = 166 × 10^(-7) (11). Free Mg2+ concentration was calculated from the chemical shifts of α- and β-ATP peaks using the MAGPAC program (28). Quantification of metabolites during flux measurements was made by averaging four spectra taken just before and just after saturation (corrected to equilibrium value for each species) and the nonsaturated spectra averaging the whole saturation period.

The forward CK reaction (PCr → ATP) was analyzed as a pseudo-first-order rate reaction. The dependence of PCr magnetization (Mpcr) as a function of the time of saturation is described by

\[
dM_{PCr}/dt = M_{qPCr}/T_{1PCr} - M_{PCr}/T_{rPCr}
\]

where M_{pcr} is the intensity of PCr magnetization in the absence of saturation and M_{qpcr} is the intensity of PCr magnetization when γ-ATP is saturated during time t. The fit of the relative PCr magnetization, M_{pcr}/M_{o}, as a function of time of saturation t, allows the determination of k_{PCr}, which is 1/T_{1PCr} + k_{PCr} (T_{1PCr} = intrinsic relaxation of PCr and T_{rPCr} = relaxation time constant) as described previously (4). The forward CK flux = k_{PCr} [PCr] is expressed in micromoles per second. The T_{1PCr} value was similar in all groups: control, 3.1 ± 0.2 (n = 12); 2DG-1, 2.9 ± 0.2 (n = 18); 2DG-2, 2.8 ± 0.2 (n = 18); and 2DG-2a, 3.1 ± 0.4 s (n = 5).

Predicted velocity of CK in myocardium. To check whether the dependence of CK flux on PCr concentration could be understood by the hypothesis of all CK isoforms functioning at equilibrium, we compared for each heart the NMR-measured velocity with the velocity expected from the well-known equilibrium behavior of MM-CK in vitro. The forward CK reaction is a rapid equilibrium random mechanism at neutral pH. The steady-state velocity of the reaction, v, relative to v_{max} is described by

\[
v = v_{max} \cdot \left( \frac{[MgADP] \cdot [PCr] \cdot K_{iADP} \cdot K_{iPCr}}{[MgATP] \cdot K_{iATP}} \right)
\]

where D is a function of the association (K_{i}), dissociation (K_{d}), and inhibitory (K_{i}) constants of each metabolite for the various enzyme complexes

\[
D = 1 + \frac{[MgADP]}{K_{iADP}} + \frac{[PCr]}{K_{iPCr}} + \frac{[Cr]}{K_{iCr}} + \frac{[MgATP]}{K_{iATP}}
\]

Thus, for each heart, the metabolite concentrations measured by NMR were used to predict the reaction velocity expected if myocardial CK isoforms function at equilibrium as CK in dilute solution in vitro. The maximal CK activity, v_{max} was constant in this acute model. v_{max} was 94.5 mM/s as
estimated from the total CK activity (1,150 IU/g wet wt measured at 30°C), assuming a $Q_{10}$ of 2.4 and a cytosolic volume of 0.435 ml H$_2$O/g wet wt. The constants used for prediction were ($\text{in mM}$) $K_m$:PCr = 1.11, Cr = 3.98; K$_D$:ADP = 0.35; Cr = 3.9; ATP = 1.54; ATP = 3.5; and K$_D$:Cr = 58, PCr = 3.9 according to McFarland et al. (14). No correction was applied for the influence of $H^+$ or Mg$^{2+}$ (see RESULTS). The amount of free enzyme, i.e., nonsaturated by its substrates, can be computed as 1/D and was expressed as percentage of total enzyme.

Oxygen Consumption

Parallel experiments were performed outside of the magnet to estimate oxygen consumption ($Q_{O_2}$) in relation to contractility in the same conditions of perfusion. "Arterial" $PO_2$ just above the aorta and "venous" $PO_2$ in the pulmonary artery were measured in-line through two flow cells, Clark electrodes, and oxymeters (Strathkelvin Inst., Glasgow, UK). $Q_{O_2}$ ("arterial" $PO_2$ – "venous" $PO_2$) was expressed in micromoles of O$_2$ per minute per gram wet weight. After equilibration, hearts were first submitted to stepwise increase in balloon volume until isovolumic conditions were reached to define the relationship between work and $Q_{O_2}$ in our control conditions. Next, hearts were submitted to protocol 2DG-1 or 2DG-2. For protocol 2DG-1, hearts were perfused with 2 mM 2DG in the presence of insulin for 6, 8, or 10 min ($n = 4, 4, 5$, respectively). For protocol 2DG-2, five hearts were perfused for 15 min in 5 mM 2DG in the presence of insulin, followed by 15 min of 2DG-free perfusate containing insulin. Steady state was obtained for both protocols 10 min after removal of insulin in the presence of 0.075 mM 2DG. Hearts were freeze clamped for ATP, PCr, and Cr biochemical determination at the end of the experiment to ensure that PCr and ATP content were similar to the NMR series. ATP synthesis of the NMR-perfused hearts was estimated from the relationship between RPP and oxygen consumption and a phosphate-to-oxygen ratio of 3.

Statistical Analysis

All results are expressed as means ± SE. Differences between groups were analyzed by analysis of variance and Student-Newman-Keuls test, except for the effect of acidosis, which was analyzed by paired t-test.

RESULTS

Metabolic and Contractile Characteristics of Hearts

The initial contractile performance for each group and the steady-state contractility during the performance of saturation transfer are summarized in Table 1 for control hearts ($n = 12$) and for 2DG protocols at 50% decrease in PCr content, including the high-ATP protocol (2DG-1, $n = 8$), the low-ATP protocol (2DG-2, $n = 9$), and the low-ATP protocol at constant pH (2DG-2a, $n = 5$). The various 2DG protocols affected neither EDP nor the coronary pressure (not shown). As previously described, a massive decrease in ATP and PCr content resulted in moderate (although significant) change in contractility in this normoxic model. When PCr was 50% of its control content, RPP was similar in protocols 2DG-1 and 2DG-2 (80 ± 3% and 77 ± 4% of initial control value, respectively; Table 1). When PCr varied from 90 to 30%, RPP ranged from 93 to 70% of initial control in protocol 2DG-1 and from 100 to 60% in protocol 2DG-2. Recovery of $pH_i$ induced by external alkalization after 2DG perfusion restored contractility to its initial control value (i.e., RPP increased by 37% in 2DG-2a). A similar increase in contractility was observed on external alkalization in two control hearts (+30%, not shown). In all protocols $P_i$ content remained low, as previously described (7).

In some hearts of the 2DG groups with 50% PCr, metabolite contents were checked biochemically at the end of the experiment (Table 2). Cr leakage occurred in two control hearts ($n = 4$). In all protocols $P_i$ content remained low, as previously described (7).

Validation of Experimental Model: Steady-State Normoxic 50% Decrease in PCr Induced by 2DG Perfusion (Protocol 2DG-1)

Figure 1 shows a typical 2DG-1 protocol leading to a steady-state decrease in PCr to 50% of its initial value.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>ATP (mmol/L)</th>
<th>PCr (mmol/L)</th>
<th>Cr (mmol/L)</th>
<th>Total Cr (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>22 ± 1</td>
<td>41 ± 3</td>
<td>25 ± 3</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>2DG-1</td>
<td>4</td>
<td>16 ± 4</td>
<td>23 ± 4$^*$</td>
<td>44 ± 5$^*$</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>2DG-2</td>
<td>9</td>
<td>8 ± 1t</td>
<td>22 ± 3t</td>
<td>38 ± 2$^*$</td>
<td>60 ± 2</td>
</tr>
</tbody>
</table>

Metabolite content values are means ± SE in nanomoles per milligram protein; $n$, no. of hearts. Cr, creatine. Different from control: *$P < 0.05$; †$P < 0.001$.
content and the time course of changes in contractile activity and phosphorus contents. At the onset of 1 mM 2DG perfusion in the presence of insulin, a transient biphasic change in contractile activity was observed. Infusion of 0.075 mM 2DG in the control perfusate medium stabilized contractility and metabolite contents. The rate of 2DG phosphorylation used to counterbalance 2DG dephosphorylation (0.016 mmol·mg protein⁻¹·min⁻¹) is four orders of magnitude smaller than CK flux and is thus negligible in terms of ATP consumption. The new steady state in metabolites allowing steady-state CK flux determination lasted at least 80 min.

Influence of 50% Decrease in PCr Content on Forward Kinetics of CK

Figure 2A shows spectra representative of the metabolite contents of the initial control and of the new steady state in three individual hearts of the control, 2DG-1, and 2DG-2 groups. A saturation-transfer experiment is performed for a typical 2DG-1 heart at 50% PCr (Fig. 2B). Longer saturation of ATP resulted in a progressive decrease in the observed magnetization of PCr as a result of both CK activity and the PCr relaxation process. The behavior of the relative PCr magnetization (M_r/M_o) is plotted as a function of the time of saturation in control and 2DG-1 (Fig. 2C). The decrease in PCr magnetization as a function of time of saturation is markedly accelerated at low PCr content: k_f increased from 0.49 in control to 0.85 s⁻¹ in 2DG-1. Figure 3 summarizes the PCr, ATP, and free ADP concentrations and pH_i for control hearts and when PCr was reduced by 50%. In 2DG-1, ATP decreased to 60% of its control (P < 0.01) and Cr rose from 9.7 ± 0.3 to 16.3 ± 0.4 mM (not shown; P < 0.001). Consequently, the calculated free ADP was two times higher in 2DG-1 (72 ± 5 µM) than in control (38 ± 3 µM, P < 0.001). The kinetics of CK are shown for the same hearts in Fig. 4. The pseudo-first-order apparent rate constant k_f was two times higher in 2DG-1 than in control (0.82 ± 0.07 vs. 0.46 ± 0.02 s⁻¹; P < 0.001). As a result, CK flux remained similar in 2DG-1 and in control (6.3 ± 0.6 and 6.5 ± 0.2 mM/s, respectively). Although, as already described, contractile activity was significantly reduced compared with each heart initial value (Table 1), the difference between the absolute contractile value of the control and that of the 2DG-1 group did not reach significance.

Influence of Variation in PCr Content on Kinetics of CK Forward Flux in 2DG

A large range of steady-state levels of PCr (from 95 to 30% of control) was generated to check the dependence of CK flux on [PCr]. Concomitantly, ATP concentration decreased from 5 to 3.3 mM and free ADP rose from 25 to 116 µM. As [PCr] decreased, k_f increased from 0.38 to 1.29 s⁻¹ (Fig. 5A,a). The forward CK flux was again independent of [PCr] (Fig. 5A,b); its mean value, 5.6 ± 0.3 mM/s (n = 18), was similar to control. Thus, because of an increase in k_f, CK flux was sustained for a threefold change in PCr (and Cr) content.

Sensitivity of CK Kinetics to Change in Adenylate Content

Because of the CK equilibrium, a modification of [PCr] is always associated with variations in [ADP] and [ATP]. To identify the influence of adenylates on CK flux, we generated an experimental situation (protocol 2DG-2, see EXPERIMENTAL PHYSIOLOGICAL GROUPS) in which the PCr content was 50% as in 2DG-1 but the ATP was decreased to 27 ± 3% of control, leading to an [ADP] similar to that found in the control group (Fig. 3). Figure 4 shows that k_f was still higher than in control (k_f = 0.72 ± 0.08 s⁻¹, P < 0.05). Although CK flux...
Fig. 2. Representative spectra and analysis of saturation-transfer experiment. A: representative spectra of control, 2DG-1, and 2DG-2 protocols corresponding to initial steady state and to period of saturation transfer. Each trace is sum of 2 spectra (for saturation, trace is sum of 1 spectrum before and 1 spectrum after saturation-transfer experiment). B: progressive saturation-transfer experiments in a representative heart of 2DG-1 group: γ-ATP is saturated for various times shown. For each spectrum, delay was adjusted so that interpulse duration was 10 s (no. of scans = 40). Intensity of PCr decreases with time of saturation of γ-ATP. Saturation at mirror frequency did not affect PCr (not shown). C: analysis of rate constant $k_f$. $k_f$ increases with decrease in PCr content. Each curve is fitted by $1/t = 1/T_1 + k$, where $t$ is relaxation time constant and $T_1$ is intrinsic relaxation of PCr. For a representative control, saturation of γ-ATP was performed for 0, 0.5, 0.8, 1.2, 2.0, 4, and 9 s; $T_1 = 3.34$ s, $k = 0.48$ s$^{-1}$. For 2DG-1 (heart shown in B), $T_1 = 2.84$ s, $k = 0.85$ s$^{-1}$.

Fig. 3. Nuclear magnetic resonance (NMR)-measured metabolite contents in control (open bars; $n = 12$) and in 2DG-1 (solid bars; $n = 8$), 2DG-2 (striped bars; $n = 9$), and 2DG-2a (crosshatched bars; $n = 5$) protocols at 50% PCr content. ADP$_f$, free ADP calculated under hypothesis of CK equilibrium. Difference from control: ***$P < 0.01$; ****$P < 0.001$. Effect of adenylate depletion, difference from 2DG-1: #P < 0.05; ###P < 0.001.
moderate compared with protocol 2DG-1. Thus, in this group characterized by low ATP and normal ADP contents, CK flux has a tendency to decrease together with PCr content (Fig. 5B, b). This was confirmed by comparing two groups of hearts at 35% PCr content in protocols 2DG-1 and 2DG-2. In 2DG-1 (PCr = 5.3 ± 0.3 mM, ATP = 4.3 ± 0.47 mM, and ADP = 95 ± 7 µM), CK flux was similar to control (5.1 ± 0.5 mM/s, n = 4). However, in 2DG-2 (identical PCr concentration = 5.2 ± 0.2 mM but ATP = 1.6 ± 0.2 mM and ADP = 34 ± 16 µM), CK flux (3.1 ± 0.2 mM/s, n = 6) appeared significantly decreased in comparison to both control and 2DG-1 (P < 0.05). Thus at a low adenylate content a decrease in PCr impaired CK flux.

Influence of pH on CK Kinetics at Low ATP

2DG induced intracellular acidosis in protocols 2DG-1 and 2DG-2 (Fig. 3). We checked the eventual influence of this acidosis on CK kinetics in protocol 2DG-2 by changing the pH. At steady-state metabolite contents (50% PCr at low ATP) pH was 6.98 ± 0.01 (n = 5). By increasing pHo to 7.65, we restored pH to the control value, 7.14 ± 0.01. Despite the increased contrac-
tivity (Table 1), neither PCr and ATP contents nor $k_1$ changed significantly. CK flux was thus similar at $pH_0$ 7.35 and 7.65 (4.9 ± 0.7 and 4.8 ± 0.5 mM/s, respectively; n = 5). Similar results were observed in two control hearts perfused at $pH_0$ 7.65 (not shown). Thus this degree of acidosis does not account for the decrease in CK flux observed at low adenylate content.

Relationship Between ATP Synthesis and CK Flux

To estimate ATP synthesis flux, we determined the relation between $Q_{O_2}$ and contractility in parallel experiments. All metabolite contents were similar to those of the NMR series (shown in Fig. 3). For example, in protocol 2DG-2, PCr, ATP, and Cr contents measured at the end of the $Q_{O_2}$ experiment were 22.6 ± 1.3, 7.1 ± 0.4, and 42.8 ± 2.3 nmol/mg protein, respectively (n = 5). $Q_{O_2}$ and contractility are given in Table 3 for each series for control perfusion and at the new steady state. None of the 2DG perfusion protocols affected the control of each series (steady state).

Table 3. Oxygen consumption and contractility during control and steady-state metabolite depletion in protocols 2DG-1 and 2DG-2

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Control of Each Series</th>
<th>Experimental Condition (steady state)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RPP</td>
<td>$Q_{O_2}$</td>
</tr>
<tr>
<td>2DG-1 6 min</td>
<td>4</td>
<td>3.5 ± 0.4</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td>2DG-1 7 min</td>
<td>4</td>
<td>3.3 ± 0.3</td>
<td>8.7 ± 0.2</td>
</tr>
<tr>
<td>2DG-2 10 min</td>
<td>4</td>
<td>3.2 ± 0.1</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>2DG-2 15 min</td>
<td>5</td>
<td>2.9 ± 0.2</td>
<td>7.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; from parallel experiments performed outside of magnet; n, no. of hearts. $Q_{O_2}$, oxygen consumption in micromoles $O_2$ per gram wet weight per minute; 2DG-1, 2 mM 2DG + insulin followed by infusion of 0.075 mM 2DG (15 min); 2DG-2, 5 mM 2DG + insulin (15 min) followed by 2DG free + insulin (15 min) and infusion of 0.075 mM 2DG (15 min). RPP is expressed as $10^4$ mmHg·beat·min$^{-1}$. $Q_{O_2}$ in 10$^6$ mmHg·g·beat·min$^{-1}$.

Comparison of NMR-Measured Flux and Flux Predicted in Hypothesis of All CK Isoforms at Equilibrium

The velocity $v$ of CK was predicted for each heart from the in vitro kinetic constants of the enzyme, the NMR-measured concentrations of PCr, ATP, and $H^+$, and the free ADP calculated by the CK-equilibrium hypothesis (see Metabolite concentrations and NMR-measured forward flux of CK). Table 5 shows the comparison of the predicted and the NMR-measured velocity in the various protocols. For control hearts, the predicted $v$ was 14.5 ± 6 (n = 12) and the NMR-measured value was 6.5 ± 2 mmol/s. This difference, although highly significant, should be interpreted with caution because it relies on several assumptions (see Dependence of CK Flux on Concentrations of CK Substrates and Products at Low Adenylate Content). The deviation from prediction markedly increased in all 2DG groups (Table 5). The predicted flux was threefold higher than actually measured by NMR in the 2DG-1 group and even fivefold higher at low PCr and adenylate content (2DG-2 35% PCr group).

Table 4. Creatine kinase and ATP synthesis fluxes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>CK Flux</th>
<th>Oxygen Consumption</th>
<th>ATP Synthesis</th>
<th>CK Flux/ATP Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>17.7 ± 0.6</td>
<td>9.0 ± 0.6</td>
<td>5.6 ± 0.4</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>2DG-1 50% PCr</td>
<td>8</td>
<td>17.1 ± 1.6</td>
<td>7.4 ± 0.5</td>
<td>4.6 ± 0.3</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>2DG-2 50% PCr</td>
<td>9</td>
<td>14.4 ± 1.8</td>
<td>6.9 ± 0.4*</td>
<td>4.3 ± 0.3*</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>2DG-1 35% PCr</td>
<td>4</td>
<td>13.9 ± 1.4</td>
<td>8.8 ± 1.1</td>
<td>5.5 ± 0.7</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>2DG-2 35% PCr</td>
<td>6</td>
<td>8.4 ± 0.6*</td>
<td>6.3 ± 0.6*</td>
<td>4.0 ± 0.4*</td>
<td>2.3 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. NMR measured creatine kinase (CK) flux in nanomoles per milligram protein per second. Oxygen consumption is estimated from relationship $Q_{O_2}$ in $\mu$mol·mg protein$^{-1}·s^{-1}$ · wet wt$^{-1}$ = 2.377·RPP + 0.625. ATP synthesis (in nmol·mg protein$^{-1}·s^{-1}$) was calculated assuming P/O ratio of 3. Different from control: $\ast P < 0.05; \dagger P < 0.01$. Effect of adenylate depletion: different from 2DG-1, $\ddagger P < 0.05$.

Table 5. Comparison of measured and predicted velocity of CK forward reaction

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>NMR-Measured Velocity, mmol/s</th>
<th>Predicted Velocity, mmol/s</th>
<th>Deviation from Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>6.5 ± 0.2</td>
<td>14.5 ± 0.6</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>2DG-1 (50%PCr)</td>
<td>9</td>
<td>6.3 ± 0.6</td>
<td>19.0 ± 0.5</td>
<td>3.3 ± 0.2*</td>
</tr>
<tr>
<td>2DG-2 (50%PCr)</td>
<td>9</td>
<td>5.3 ± 0.7</td>
<td>14.6 ± 0.7</td>
<td>3.1 ± 0.4*</td>
</tr>
<tr>
<td>2DG-1 (35%PCr)</td>
<td>4</td>
<td>5.1 ± 0.5</td>
<td>20.3 ± 0.6</td>
<td>4.1 ± 0.6*</td>
</tr>
<tr>
<td>2DG-2 (35%PCr)</td>
<td>6</td>
<td>3.1 ± 0.2*</td>
<td>14.9 ± 0.9</td>
<td>5.0 ± 0.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. Predicted velocity, $v$ calculated under hypothesis of all CK isoforms at equilibrium using in vitro MM-CK kinetic constants of McFarland et al. (14) and NMR-measured concentrations of metabolites for each heart (see MATERIALS AND METHODS); deviation from prediction, $v$ predicted/ measured by saturation transfer. 2DG-1 and 2DG-2 (50% PCr) correspond to groups shown in Figs. 4 and 5. 2DG-1 and 2DG-2 (35% PCr) hearts are presented in Tables 1 and 4. All predicted fluxes were different from measured flux ($P < 0.001$). Different from control: $\ast P < 0.05; \dagger P < 0.01; \ddagger P < 0.001$. 
DISCUSSION

Using an acute normoxic model of adenylate and PCr depletion, with long-term steady state of contractile activity and metabolite contents, we have measured in the isolated perfused heart the kinetics of the CK reaction under a wide range of concentrations of its substrates and products. Furthermore, the experiment was designed to separate the respective influence of the adenylate and guanidylate pools on CK velocity. The main importance of this study was to provide an acute model in which the dependence of CK kinetics on PCr and ATP concentrations can be analyzed, without change in the other factors that are known to influence CK activity in the pathophysiological cardiac muscle. These include total CK activity, isozymic CK distribution, and remodeling of the myocardial cell. 

Characteristics of Model

The experimental evidence of a sustained CK flux despite wide variations of its substrates confirms the hypothesis we put forward to explain sustained contractility in our normoxic model of adenylate depletion (7). As previously discussed, sustained ATP synthesis is expected from the presence of abundant oxygen and nonglycolytic substrate. The decrease in ATP-to-ADP ratio and/or the rise in ADP would promote rather than inhibit respiration of isolated mitochondria. ATP does not limit ATPase activity, because even at its lower cytosolic concentration it is still two orders of magnitude higher than the reported $K_m$ of the ATPases. Thus sustained myofibrillar and mitochondrial functions were understood, and we hypothesized that transcytosomic transport of substrate should also be normal. Here we demonstrate experimentally the validity of this hypothesis. Wide variations in CK substrate concentrations induced minimal changes in CK flux, which in all cases remained much higher than the ATP synthesis rate and should not limit energy transfer in the cell. 

We can apply these findings to the understanding of other physiological perturbations. For example, in the isolated perfused heart, severe hypoxia induces the same variation in metabolite contents as in protocol 2DG-1, namely a moderate change in ATP, a marked decrease in PCr, and a rise in Cr and ADP contents. However, it results in a strong impairment of CK flux (24). Because CK flux in 2DG-1 is normal, the hypoxic low CK flux cannot be explained by kinetic factors resulting from changes in CK metabolite contents. Moreover, in vivo, when the animal breathes hypoxic mixtures, a similar reduction in CK flux occurs even in the absence of significant alterations in ATP and PCr contents (3). In the absence of kinetic factors, the origin of hypoxic impairment of CK flux is still not fully understood. Further work is needed to study the respective roles of an inhibition of mitochondrial CK flux, of a defect in oxygenation leading to cellular heterogeneity in the myocardium, or of the accumulation of some unknown cytosolic factor inhibiting CK.

Insensitivity of CK Flux to Variation in Concentration of CK Substrates at High Adenylate Content

The insensitivity of myocardial forward CK flux to a wide variation in PCr, Cr and ADP at high adenylate content (Fig. 4) is in agreement with the known behavior of MM-CK in dilute solution in vitro (9). In skeletal muscle a threefold change in PCr content at constant ATP can be induced by a transition from rest to high stimulation rates. CK flux remained constant despite a 10-fold increase in ATP synthesis rate. (10, 14, 21). Thus in skeletal muscle the stability of CK flux, which is expected from the kinetics of CK in vitro, has been interpreted as a proof of the equilibrium of CK at all points of the cell, in agreement with the predominance of the buffering role of CK in this type of muscle (15).

In myocardium it is not as straightforward to study the dependence of flux towards CK metabolites because the cardiac specificity, as opposed to skeletal muscle, is to adjust its contractility with minor changes in high-energy phosphate concentrations. Two experimental models have been previously used, transition from the KCl-arrested heart to maximal work and a chronic model of creatine depletion induced by feeding the animal analogs of Cr [e.g., guadinopropionic acid (GPA)]. In the former, on transition from arrest to maximal systolic activity, [PCr] decreased by at most 30%, and the CK forward flux was enhanced by 30-50% (4, 9, 12). However, as seen from analysis of Fig. 3 in Bittl and Ingwall (4), the main increase in CK flux occurs during the transition from arrest to low-work conditions. Since that report it has been recognized that the KCl-arrested heart is a puzzling case in which the set of kinetic constants that predict CK flux in hearts performing work cannot be used (13, 16). In the chronic GPA-fed animal model, the massive decrease of both Cr and PCr occurs without major change in CK flux (22). Our deoxyglucose model confirms these results in an acute model in which the influence of Cr and PCr content on CK velocity can be analyzed in the absence of the numerous adaptations in glycolytic pathway, mitochondrial function, isomyosin profile, and thus in the economy of contraction found in chronic GPA-fed animals.

Dependence of CK Flux on Concentrations of CK Substrates and Products at Low Adenylate Content

In none of the previous models of cardiac or skeletal muscle could the influence of adenylate content on CK velocity be studied because ATP levels remained high. The low adenylate content consistently observed in models of ischemia and on reperfusion is due to imbalance of ATP synthesis and utilization and is often associated with a detachment of mito-CK or a decrease in its activity due to a reduced oxidative phosphorylation. Our 2DG model offers a unique opportunity to study the influence of wide change in adenylate content independently of an alteration of ATP synthesis flux.
CK flux remained constant for a twofold reduction in ATP content (protocol 2DG-1) in agreement with in vitro analysis (9); however, the impaired CK flux observed at lower adenylate content (protocol 2DG-2, Fig. 5B) was not previously observed. In the hypothesis of the CK equilibrium, such sensitivity should result from a change in the concentrations of PCr, Cr, ATP, and ADP or in ionic content of H⁺ or Mg²⁺. Let us compare the metabolite and ionic contents in the 2DG-1 and 2DG-2 groups at 50% PCr content (Fig. 3). The design of the experiment excludes the role of PCr and Cr because their contents were chosen to be identical. In vitro CK flux is well known to be modulated by H⁺ and Mg²⁺ concentration (9, 11). The CK flux is identical in 2DG-2 and 2DG-2a, in which all metabolite contents except H⁺ are similar. This insensitivity of CK to small pH changes (0.2 pH unit) agrees with previous data (11). In ischemia, ATP depletion is associated with an accumulation of free Mg²⁺; such is not the case in our deoxyglucose model, most probably because of the preservation of mitochondrial function and ionic gradients. Free Mg²⁺ was similar in control, 2DG-1, and 2DG-2 groups [0.31 ± 0.01 (n = 9), 0.35 ± 0.01 (n = 12), and 0.31 ± 0.02 mM (n = 10), respectively]. Marked alterations of free ADP (from 70 µM in 2DG-1 to 30 µM in 2DG-2) are in the range expected to influence CK flux (see Predicted velocity of CK in myocardium). However, because in control conditions maximal CK flux was obtained for an ADP content of 38 µM, i.e., similar to 2DG-2, the decreased ADP content should not account for the impairment of CK flux observed in 2DG-2. This is confirmed by the comparison of the NMR-measured velocity and the theoretical velocity predicted from the in vitro kinetics of CK in dilute solution. In the absence of cellular compartmentation of CK metabolites and if all CK isoforms function at equilibrium, the velocity of myocardial CK must be governed by the NMR-measured concentrations of ATP, PCr, Cr, H⁺, and Mg²⁺ and by the free ADP concentration calculated assuming CK equilibrium. In control perfusion, the NMR-measured flux was 50% lower than predicted from the in vitro kinetic analysis (Table 5) using the set of constants of McFarland et al. (14). It should be noted that the different sets of CK kinetic constants (1, 9, 13, 14) result in a wide range of CK theoretical velocity (for our control hearts from 6 to 20 mM/s). However, for any set of constants the discrepancy between measured and theoretical velocity consistently increased at low PCr and adenylate content. Several alternative hypothesis could explain this disparity. First, in vivo CK cellular activity might be modulated by cytosolic factors other than CK metabolites; second, the flux of the CK-bound isozymes may not be governed by the bulk cytosolic metabolite concentrations; and third, the concentration of active enzyme might vary. On the basis of modeling, planar anions (mainly chloride and bicarbonate) have been suggested to decrease in vivo CK by stabilizing the dead-end complexes of the “enzyme·Cr·MgADP” (14, 17). This hypothesis, based purely on modeling, definitely requires experimental validation, because such inhibition would potentially be a very important regulatory mechanism of cellular CK flux in view of the relative insensitivity of CK to its substrate and products. However, we do not believe an increase in “planar anion inhibition of CK” to be a major determinant of the impaired CK flux in our 2DG model, because respiration, contractility, and electrical activities are close to normal.

Alternatively, the NMR-measured metabolite content might not represent the concentration at the active sites of the enzyme(s). In other words, the velocity of CK-bound isozymes could be controlled by metabolite concentrations different from the bulk cytosol concentrations. Even without any physical compartmentation the vicinity of two enzymes is known to affect their kinetics, as illustrated in vitro by the alterations of the apparent kinetic properties of myosin when coimmobilized with CK on artificial membranes (2). Similarly, in skinned fiber preparations, the apparent kinetics of myofibrillar ATPase are changed by the activity of MM-CK (26), and oxidative phosphorylation drives mito-CK out of equilibrium (20). Until now we considered the CK at equilibrium at any point in the cell and neglected the implication for cell function of CK bound to intracellular structures. We showed that the velocity of the enzyme cannot be fully predicted in this simplified theory, and it is therefore worth examining the hypothesis of metabolite compartmentation. Can the analysis of the NMR data give any information for this question? The design of the NMR determination of CK flux considers CK as a pseudo-first-order reaction. CK is well known to be a higher-order reaction. It follows that, first, the NMR-measured apparent rate constant, k₅, does not directly measure the rate constant of the CK reaction (K₅). Second, k₅ values should depend on the concentration of ATP, the other substrate of CK forward flux; k₅ indeed varied in the various protocols (Table 5, Figs. 4 and 5). Figure 6A shows the dependence of k₆ on the free ADP concentration calculated in the hypothesis of CK equilibrium. For the control and 2DG-1 groups, k₅ is linearly related to ADP. For each heart one can compute k₆ = k₅ [ADP]⁻¹, which should be proportional to the true rate constant of the enzyme. As expected, k₆ is indeed a constant independent of ADP (Fig. 6B); its value was 12.5 ± 0.9 s⁻¹·mM⁻¹ in control hearts and was similar for all 2DG-1 hearts (11.8 ± 0.9 s⁻¹·mM⁻¹, n = 18). As previously observed (16), the influence of H⁺ on the rate constant is minor compared with that of ADP (not shown). However, at low ATP content (2DG-2), the measured rate constant k₆ is higher than expected from the ADP concentration: most hearts are outside the 95% confidence interval (Fig. 6A). Estimation of k₅ in both 2DG-2 and 2DG-2a groups also yields higher values than found in control conditions [23.7 ± 2.4 (n = 18) and 19.1 ± 2.5 s⁻¹·mM⁻¹ (n = 5), respectively]. This deviation is clearly seen in Fig. 6B. Such a failure to observe a constant k₅ has already been reported in the failing cardiomyopathic...
The discrepancy of both $k_f$ and $k'_f$ in the ATP-depleted groups suggests that the ADP calculated from the CK equilibrium might not adequately reflect the ADP in vicinity of the enzyme(s). Why would this phenomenon influence CK flux only at low cytosolic adenylate concentration? We hypothesize that at high cytosolic ATP, the function of bound CKs, whether or not they are at the same chemical potential, might not visibly influence the global NMR-measured CK flux, which is dominated by the equilibrium behavior of cytosolic CK. However, this might not be the case at low cytosolic adenylate content. Indeed, the distribution of ATP and ADP between cytosol and mitochondria should markedly differ from control in protocol 2DG-2. Thus we propose that at low adenylate content, CK bound to intracellular structures exposed to metabolite concentrations different from bulk concentrations could become predominant in the NMR-measured flux. This hypothesis is currently under investigation by measuring, by cell fractionation (23), the distribution of metabolites between cytosol and mitochondria and by computing the flux using a model of mito-CK coupled to translocase (1).

Finally, because CK reaction velocity depends on the active enzyme concentration (i.e., the amount of enzyme saturated with substrates), an increasing proportion of free enzyme would decrease CK flux. In heart the total CK concentration is 7.5 IU/mg protein and mito-CK accounts for 30% of total CK. Thus with a specific activity of ~300 IU/mg CK, the concentration of nonmitochondrial CK is ~150 µM, i.e., highly concentrated compared with ADP. One cannot currently exclude the possibility that, as adenylate content decreases, an increasing proportion of free cytosolic CK might not be fully saturated by ADP. Furthermore, in this case, CK might not be governed by classical Michaelis-Menten kinetics. Free enzyme (see Predicted velocity of CK in myocardium) was estimated to be 4.3 ± 0.1% of total enzyme in controls and 5.9 ± 0.2% in DG-1. The increase in free enzyme in DG-2 (12 ± 1%) was, however, not sufficient to fully account for the impairment of CK flux.

Methodological Considerations

In dilute solution, in vitro, the rate of exchange of phosphoryl group catalyzed by MM-CK is the rate-determining step in the CK reaction and thus the exchange rate between PCr and γ-ATP adequately represents the CK flux (8). In vivo, because PCr is the substrate only for CK, the determination of the forward rate by conventional one-site saturation (ST) avoids the contamination by concurrent reactions occurring when the reverse flux is measured. ST, as a steady-state technique, allows equilibration among the exchanging spins during the saturation pulse and should detect exchange between all metabolite pools. Such might not be the case when rapid labeling methods are used, because they have been suggested to minimize the contribution of small intracellular CK pools (14). Several questions still arise in relation with the subcellular compartments of metabolites and CK isozymes. First, the calculation of CK flux from magnetization-transfer experiments assumes that all reactants are NMR visible and have spatially invariant T1. Such is the case for PCr; however, two pools of ATP with widely different T1 values have been observed in the isolated perfused heart (25). Thus the saturation of γ-ATP under standard magnetization-transfer conditions might not be as efficient in all subcellular compartments. As previously suggested (30), the calculation of CK flux would be more complex than generally assumed. Finally, because of the poor sensitivity of NMR, flux originating from small intracellular compartments might not be detected. It is likely that in skeletal muscle mito-CK, which represents 2–6% of total CK, would be beyond the limit of NMR detection. The situation is more favorable in the myocardium because mito-CK represents ~30% of total CK. Because our limit of flux detection is ~6% (SE/mean in Table 3) the function of bound CKs, if fully NMR visible, should be detected in the myocardium.

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