Cardiac performance and creatine kinase flux during inhibition of ATP synthesis in the perfused rat heart

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Mateo, P., V. Stepanov, B. Gillet, J.-C. Beloeil and J. A. Hoerter. Cardiac performance and creatine kinase flux during inhibition of ATP synthesis in the perfused rat heart. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H308–H317, 1999.—To study the relation among mitochondrial energy supply, cardiac performance, and energy transfer through creatine kinase (CK), two acute models of inhibition of ATP synthesis were compared in the isovolumic ace-te-perfused rat heart. Similar impairments of mechanical performance (rate-pressure product, RPP) were achieved by various step-wise decreases in O2 supply (Po2 down to 20% of control) or by infusing CN (0.15–0.25 mM). The forward CK flux measured by saturation-transfer 31P NMR spectroscopy was 6.1 ± 0.4 mM/s in control hearts. Only after severe hypoxia (Po2 < 40% of control) did CK flux drop (to 1.9 ± 0.2 mM/s at Po2 = 25% of control) together with impaired systolic activity and a rise in end-diastolic pressure. In contrast, in mild hypoxia CK flux remained constant and similar to control (5.3 ± 0.5 mM/s, not significant) despite a twofold reduction in systolic activity. Similarly in all CN groups, constant CK flux was maintained for a threefold reduction in RPP, showing the absence of a relation between cardiac performance and global NMR-measured CK flux during mild ATP synthesis inhibition.

Catecholamine stress does not alter CK flux (11, 13, 19). The absence of such a relation between mechanical activity and CK flux is also observed in skeletal muscle. The latter has been interpreted as evidence of CK being at equilibrium, whereas the relation of CK flux with contractility has been analyzed in the context of bound CK in support of the CK shuttle (1, 6). As was stressed recently (30), information resulting from the use of NMR must be considered in perspective with the abundant literature describing the high organization and compartmentation of the myocardial cell and the specific behavior of the subcellular compartments.

Indeed, both in vitro and in subcellular preparations of mitochondria or myofibrils, the apparent kinetics of an enzyme are known to be altered by the proximity of another enzyme. For example, in vitro, the activity of CK in the vicinity of myosin ATPase changes the apparent kinetics of the myosin ATPase (2) and facilitates cardiac actomyosin sliding (22). Similarly, in Triton-skinned cardiac fibers, the activity of MM-CK bound to myofibrils changes the mechanical properties of both the calcium-activated and the rigor force (28). Considering mito-CK, located in close vicinity to the adenine nucleotide translocase in the intermembrane mitochondrial space, the activation of oxidative phosphorylation has been shown to drive mito-CK out of equilibrium (21). Thus, from these in vitro and subcellular observations, an inhibition of ATP synthesis and utilization should result in a modification of bound CK fluxes. If cytosolic CK is at equilibrium, the total CK flux is expected to decrease, if NMR detects the activities of both the cytosolic and the bound CK isoforms.

Three main factors have been proposed to govern the CK flux in vivo: total CK activity, CK isozymic composition, and the concentration of products and substrates. In a normoxic model of adenylate depletion [by 2-deoxyglucose (2-DG)], we previously demonstrated (23) that CK flux in the myocardium is largely independent of the concentration of its substrates and products. In this 2-DG model, the flux of ATP synthesis and ATP utilization were quasinormal and CK flux was constant for a twofold ATP and PCr decrease. Here we reconsider the effects of inhibition of ATP synthesis in relation to myocardial performance and CK flux in conditions in which ATP and PCr content, similar to the 2-DG model, should hardly affect the kinetics of the enzyme. Apparently contradictory results were observed in hearts submitted to severe hypoxia and in hearts experiencing a mild reduction of O2 supply or chemical inhibition of respiratory chain by cyanide. First, no change in CK flux occurred in the latter models despite marked...
impairment of systolic activity. On the other hand, an increase in the severity of hypoxia resulted in a coordinated decrease in CK flux and systolic activity, pointing to the absence of a universal relation between NMR-measured CK flux (energy transfer) and contractility (energy demand). Second, in severe hypoxia, impaired CK flux was associated with a marked rise in end-diastolic pressure (EDP) for a moderate increase in free ADP, suggesting a major role for the MM-bound CK in the preservation of diastolic function.

MATERIALS AND METHODS

Physiology. Animal experimentation was performed in accordance with the Helsinki accord for humane treatment of animals during experimentation. Wistar male rats (350–450 g) were anesthetized with ethyl carbamate (2 g/kg), and hearts were perfused by the Langendorff technique at a constant flow of 13.5 ml/min as previously described (23). Briefly, a latex balloon was inserted into the left ventricle (LV) and inflated with H2O to isovolumetric conditions of work. LV systolic pressure (LVP) and coronary pressure were recorded with Statham gauges and continuously monitored on a paper recorder (Brush) and on a computer (Compaq). The perfusion solution contained (in mM) 124 NaCl, 6 KCl, 1.8 CaCl2, 1 MgSO4, 1.1 mannitol, 10 Na-acetate, and 20 HEPES and was oxygenated with 100% O2. Extracellular pH (pH,e) was adjusted with NaOH to 7.35 at 36.5°C. Mean coronary pressure, LVP, EDP, and heart spontaneous frequency were analyzed on-line. The rate-pressure product (RPP, in 104 mmHg·beat·min) was used as an index of contractility reflecting the energetic demand. Control hearts (n = 7) were perfused with 100% O2; various levels of hypoxia were applied by mixing O2 and N2 with flowmeters (Aalborg). O2 was reduced to 45 (group H45, n = 4), 35 (group H35, n = 3), and 25 (group H25, n = 8) % of control. Four additional hearts were perfused with PO2 ranging from 70 to 20% of control. Partial chemical inhibition of ATP synthesis was achieved by addition of NaCN (n = 16) at low concentrations that induce a decrease in systolic activity similar to that in the hypoxic groups. From pilot experiments three concentrations of CN were selected, 0.15 (group CN1, n = 4); 0.20 (group CNII, n = 3), and 0.25 (group CNIII, n = 6) mM. Because of a marked decrease in heart rate, three additional hearts perfused at a CN concentration of 0.25 mM were paced at 170 beats/min (group CNIV). NaCN was prepared just before the experiment, and it did not alter pHo.

NMR. 31P NMR spectra were acquired at 161.93 MHz on a Bruker AM400 wide-bore magnet in 20-mm-diameter tubes. Magnetic field homogeneity was optimized using the water signal of the heart and the frequency locked on γ-ATP con- 

![Fig. 1. Evolution of NMR spectra. A: typical spectra obtained during baseline period and just before and just after saturation-transfer experiment in 2 individual hearts: representative of severe hypoxia (25% of control O2 (H25)) and 0.25 mM cyanide (CNIV) groups (no. of scans = 128, pulse interval = 2 s). SP, sugar phosphates; PCr, phosphocreatine; ppm, parts per million. B: time course of experimental protocol. After 15 min of equilibration, isovolumic conditions of work were imposed (arrow, w); 4 baseline spectra (b) were acquired 5 min later followed by a spectrum acquired at equilibrium (pulse interval = 10 s). Inhibition of respiration by hypoxia or cyanide was induced (arrow, i); after 10 min, 4 spectra were acquired just before saturation period (b.s.). Time-dependent saturation transfer (TDST) period was followed by 4 spectra (after saturation, a.s.). Heart was freeze clamped (arrow, f).]
myocardial CK flux during inhibition of ATP synthesis

The flux of CK was measured in the same heart during control and after inhibition of ATP synthesis. In this case, 16–24 scans for each spectrum were used for determination of the control flux; the values were similar to the control group. Biochemical analysis of perchloric acid extracts was performed to measure ATP, PCr, and Cr content as previously described (9). Values are expressed in nanomoles per milligram of protein. Biochemical determination confirmed that none of the experimental conditions induced Cr leakage. Cr and free ADP were thus calculated from the end of the experiment and PCr measured on each spectrum.

NMR quantification was performed with a homemade program on the area of each peak corrected for saturation. The sum of phosphorylated compounds (sum P) corresponds to all NMR-visible phosphorus signals. A biochemically determined PCr content of 43 nmol/mg protein for control hearts was used as internal standard, and cytosolic volume was taken as 2.72 µl/mg protein. Intracellular pH (pH_i) was determined from the shift of P_i with respect to PCr. FreeADP was calculated from the equilibrium of the CK reaction with the apparent equilibrium constant (K_eq) = 166 × 10^(-6) (pH = 7). Quantification of metabolites during flux measurements was made by averaging four spectra taken just before and just after saturation (corrected to their 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 (M_PCr) as a function of the time of saturation is described by

\[ \frac{dM_{PCr}}{dt} = M_{PCr} - M_{PCr}T_{1PCr}^{-1} + k_i \]

where T1_PCr is intrinsic relaxation of PCr and k_i is apparent CK forward rate constant, as described in Ref. 6. The forward CK flux is

\[ F = k_i [PCr] \]

expressed in millimolar per second. T1_PCr values were 3.2 ± 0.3 s (n = 7) in control; no significant difference was observed among the hypoxic groups (mean value 3.4 ± 0.3 s, n = 15) or among the CN groups (mean value 2.8 ± 0.3 s, n = 16). Predicted velocity of CK in myocardium. We compared for each heart the NMR-measured velocity with the velocity expected if myocardial CK isoforms function at equilibrium as CK in dilute solution in vitro. MgATP was assumed to be equivalent to total ATP because of the high affinity constant of Mg for ATP. MgATP was calculated from CK equilibrium. V_max was 94.5 mmol/s, as estimated from the total CK activity (1.510 IU/g wet wt measured at 30°C) assuming a Q10 of 2.4 and a cytosolic volume of 0.435 ml H2O/g wet wt. The constant used for prediction, taken from Ref. 1, were ([mmol/mg]: association: K_i,ADP = 0.167, K_i,PCr = 1.67, K_i,ATP = 15.5, K_i,ADP = 0.4; dissociation: K_i,ADP = 0.222, K_i,PCr = 4.73, K_i,ATP = 34.9, K_i,ATP = 0.9; and inhibitory: K_i,PCr = 34.9, K_i,PCr = 24. The amount of free enzyme (i.e., nonsaturated by its substrates) can be computed as 1/D and is expressed as percentage of total enzyme.

O2 consumption. Parallel experiments were performed outside the magnet to estimate the relation between O2 consumption (QO2) and mechanical performance in hypoxia (n = 5) or during cyanide perfusion (n = 4). Stepwise decrease in PO2 from 100 to 80, 70, 60, 50, 35, and 25% of control was induced in five hearts and stepwise change in cyanide concentrations (ranging from 0.1 to 0.25 mM) in four hearts. Steady-state parameters were measured after 10 min of stabilization in each condition. "Arterial" PO2 (PavO2) just above the aorta and "venous" PO2 (PvO2) in the pulmonary artery were measured in line through two flow cells, Clark electrodes, and oximeters (Strathkelvin Inst., Glasgow, UK). QO2([PavO2 − PavO2, in µM O2·min·g wet wt]) × 104), was linearly correlated to RPP. It was described in hypoxia by QO2 = 2.38 × RPP + 0.29 (r2 = 0.920) and in cyanide by QO2 = 1.46 × RPP + 2.21 (r2 = 0.899), with QO2 expressed in micromoles of O2 per gram of wet weight per minute and RPP expressed in 10^4 millimeters of mercury times beats per minute. Some hearts freeze clamped and analyzed for PCr and ATP contents confirmed the equivalence of the QO2 and the NMR series. Thus the relation between QO2 and RPP was used to estimate QO2 in the NMR-perfused hearts and maximal ATP synthesis assuming a P/O of 3 and a protein content of 160 mg protein/g wet weight.

Statistical analysis. All results are expressed as means ± SE. Differences between groups were analyzed by variance analysis and Student-Newman-Keuls test.

RESULTS

Cardiac performances. The initial parameters of contractility were similar in all series. The pooled values were LVP = 143 ± 3 mmHg, frequency = 226 ± 5 beats/min, RPP = 3.2 ± 0.1 × 10^4 mmHg·beat·min⁻¹, coronary pressure = 70 ± 2 mmHg, and EDP = 6 ± 1 mmHg (n = 38). Table 1 shows the averaged mechani-
Table 1. Contractile parameters of NMR hearts perfused in control, hypoxia, or cyanide

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>PaO₂</th>
<th>EDP rise</th>
<th>LVP</th>
<th>Frequency</th>
<th>RPP</th>
<th>RPP%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>100</td>
<td>-2 ± 3</td>
<td>140 ± 7</td>
<td>222 ± 8</td>
<td>3.1 ± 0.2</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>4</td>
<td>44 ± 2</td>
<td>16 ± 5</td>
<td>84 ± 6</td>
<td>212 ± 13</td>
<td>18 ± 0.1</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>H35</td>
<td>3</td>
<td>34 ± 3</td>
<td>33 ± 3</td>
<td>78 ± 10</td>
<td>178 ± 20</td>
<td>1.4 ± 0.2</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>H25</td>
<td>8</td>
<td>25 ± 1</td>
<td>58 ± 6</td>
<td>66 ± 8</td>
<td>146 ± 26</td>
<td>0.9 ± 0.1</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Cyanide</td>
<td>CNI</td>
<td>4</td>
<td>100</td>
<td>5 ± 1</td>
<td>128 ± 7</td>
<td>174 ± 16</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>CNII</td>
<td>3</td>
<td>100</td>
<td>17 ± 3</td>
<td>106 ± 3</td>
<td>84 ± 13</td>
<td>0.9 ± 0.2</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>CNIII</td>
<td>6</td>
<td>100</td>
<td>21 ± 3</td>
<td>112 ± 8</td>
<td>91 ± 10</td>
<td>1.0 ± 0.2</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>CNIV</td>
<td>3</td>
<td>100</td>
<td>19 ± 3</td>
<td>82 ± 9</td>
<td>170</td>
<td>1.4 ± 0.3</td>
<td>44 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. Frequency is expressed in beats/minute, PaO₂, in % of control. LVP, left ventricular systolic pressure (in mmHg); RPP, rate-pressure product (in 10⁴ mmHg·beat·min⁻¹); EDP rise, end-diastolic pressure rise (in mmHg; mean initial EDP for all groups = 6 mmHg); RPP% = RPP in % of initial individual control. Perfusion conditions of various groups are hypoxia: H35, H25, and H45, reduction in oxygen to 45, 35, and 25% of control, respectively, and chemical inhibition: CNI, CNII, and CNIII, perfusion with CN at concentration of 0.15, 0.20, and 0.25 mM, respectively; CNIV, 0.25 mM CN-paced hearts. Mean coronary pressure was similar in all groups (initial value = 72 mmHg); it was stable for control, H45, H35, and all cyanide groups but increased by 18 ± 2 mmHg in H25 group (P < 0.05). Significantly different from control: aP < 0.05; bP < 0.01; cP < 0.001. Comparison between CN and hypoxia groups is shown only in comparison with H25: dP < 0.05; eP < 0.01; fP < 0.001.

Table 2. NMR-measured metabolite contents in control, hypoxic, and cyanide hearts

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>PCr</th>
<th>ATP</th>
<th>P&lt;sub&gt;i&lt;/sub&gt;</th>
<th>ADP</th>
<th>Decrease in Sum P</th>
<th>pH&lt;sub&gt;i&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>141 ± 0.7</td>
<td>7.1 ± 0.2</td>
<td>3.2 ± 0.4</td>
<td>36 ± 3</td>
<td>3.1 ± 0.7</td>
<td>7.09 ± 0.01</td>
</tr>
<tr>
<td>H45</td>
<td>4</td>
<td>12.5 ± 0.6</td>
<td>5.3 ± 0.4</td>
<td>5.6 ± 0.9</td>
<td>39 ± 5</td>
<td>10.9 ± 1.0</td>
<td>7.14 ± 0.02</td>
</tr>
<tr>
<td>H35</td>
<td>3</td>
<td>9.4 ± 0.4</td>
<td>4.2 ± 0.6</td>
<td>6.1 ± 0.5</td>
<td>46 ± 6</td>
<td>9.6 ± 0.3</td>
<td>7.07 ± 0.02</td>
</tr>
<tr>
<td>H25</td>
<td>8</td>
<td>7.8 ± 0.4</td>
<td>3.7 ± 0.4</td>
<td>7.4 ± 0.5</td>
<td>53 ± 4</td>
<td>7.3 ± 0.5</td>
<td>7.09 ± 0.03</td>
</tr>
<tr>
<td>CNI</td>
<td>4</td>
<td>8.7 ± 0.7</td>
<td>6.6 ± 0.5</td>
<td>7.0 ± 1.6</td>
<td>83 ± 9</td>
<td>4.0 ± 1.4</td>
<td>7.06 ± 0.01</td>
</tr>
<tr>
<td>CNII</td>
<td>3</td>
<td>8.1 ± 0.2</td>
<td>6.9 ± 0.7</td>
<td>8.6 ± 0.3</td>
<td>91 ± 7</td>
<td>2.9 ± 1.0</td>
<td>7.07 ± 0.01</td>
</tr>
<tr>
<td>CNIII</td>
<td>6</td>
<td>5.3 ± 0.6</td>
<td>4.5 ± 0.5</td>
<td>20.8 ± 1.5</td>
<td>114 ± 23</td>
<td>3.5 ± 0.6</td>
<td>7.05 ± 0.01</td>
</tr>
<tr>
<td>CNIV</td>
<td>3</td>
<td>6.2 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>13.3 ± 1.7</td>
<td>84 ± 9</td>
<td>4.5 ± 0.4</td>
<td>7.04 ± 0.02</td>
</tr>
</tbody>
</table>

Values refer to mean ± SE metabolite contents during saturation period; n, no. of hearts. Phosphocreatine (PCr), ATP, and P<sub>i</sub> concentrations are millimolar; free ADP concentration is micromolar. Decrease in sum P, difference between initial sum of all NMR-visible phosphorus and NMR-visible phosphorus during saturation on control spectra (initial sum P = 50 ± 1 mM, n = 18); pH<sub>i</sub>, intracellular pH. Significantly different from control: aP < 0.05; bP < 0.01; cP < 0.001. Comparison between CN groups and H25 group: dP < 0.05; eP < 0.01; fP < 0.001.
Table 3. Comparison of biochemical and NMR-measured metabolite contents

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>PCR</th>
<th>ATP</th>
<th>Cr</th>
<th>Total Cr</th>
<th>PCr</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>40±2</td>
<td>23±1</td>
<td>27±4</td>
<td>65±2</td>
<td>38±1</td>
<td>19±2</td>
</tr>
<tr>
<td>H45</td>
<td>3</td>
<td>36±5</td>
<td>14±1</td>
<td>27±2</td>
<td>63±5</td>
<td>35±2</td>
<td>15±2</td>
</tr>
<tr>
<td>H25</td>
<td>4</td>
<td>18±3</td>
<td>8±2</td>
<td>45±5</td>
<td>63±6</td>
<td>20±1</td>
<td>8±1</td>
</tr>
<tr>
<td>CN1</td>
<td>3</td>
<td>23±3</td>
<td>18±3</td>
<td>36±4</td>
<td>61±5</td>
<td>24±1</td>
<td>17±2</td>
</tr>
</tbody>
</table>

Values (in nmol/mg protein) are means ± SE; n, no. of hearts. Biochemical determination of metabolites at end of experiment (freeze-clamped hearts) is compared with NMR contents (determined on last spectra). Cr, creatine.

(Fig. 2A). The exponential decrease in the relative PCR magnetization (Mz/Mo) is plotted as a function of the time of saturation of γ-ATP in representative hearts of the control, H25, and CNIV groups (Fig. 2B). This decrease was markedly slowed down by hypoxia and accelerated by cyanide.

Figure 3 summarizes the change in the dynamic parameters kᵣ and CK flux for the various groups. The apparent rate constant of CK forward flux, kᵣ, was 0.43 ± 0.02 s⁻¹ (n = 7) in control. It was similar in H45 and H35 [0.48 ± 0.07 (n = 4) and 0.34 ± 0.07 s⁻¹ (n = 3), respectively] and decreased significantly in severe hypoxia [0.28 ± 0.02 in H25 (n = 8); P < 0.05]. As a result of changes in both PCR and kᵣ, CK flux remained similar to control in moderate hypoxia (5.5 ± 0.4 mM/s in H45 vs. 6.1 ± 0.4 mM/s in control). For more severe hypoxic conditions, CK flux progressively decreased. CK flux reached one-half of its control value in H35 (3.1 ± 0.5 mM/s, P < 0.01) and one-third in H25 (1.9 ± 0.2 mM/s, P < 0.001). Paired analysis of CK flux performed in the same heart in normoxia and hypoxia (ranging from 20 to 70% of control O₂; n = 8) also illustrates the constancy of CK flux for a moderate restriction in O₂ supply and its progressive inhibition in severe hypoxia (Fig. 4). Even in severe hypoxia, there was no significant magnetization transfer from γ- to β-ATP, a sign of activation of the adenylate kinase exchange.

In all cyanide experiments, the rate constant kᵣ was significantly increased to a maximal value of 1.1 ± 0.1 s⁻¹ (Fig. 3). As a result of the opposite change in kᵣ and PCR content, CK flux remained constant (6.0 ± 0.4, 6.4 ± 1.1, 5.7 ± 0.3, and 5.8 ± 0.5 mM/s in CN1–CNIV, respectively) and similar to control. This constancy was also observed with paired analysis of CK flux in the same heart in the presence or absence of cyanide (Fig. 4, n = 5). Because we focused on the relation between energetic demand (mechanical performance) and CK flux, we only used low cyanide concentrations. As expected, higher concentrations (1–2 mM), provoking full inhibition of oxidative metabolism and cardiac arrest, decreased CK flux (not shown).

CK flux and metabolite contents. The dependence of the forward CK flux on the PCR content was analyzed in individual hearts. In hypoxia, both the apparent rate constant and CK flux (Fig. 5) progressively decreased with PCR. With cyanide, however, for the same range of PCR content (from 10.5 to 5.5 mM), CK flux was independent of PCR. The velocity of CK obviously depends on the concentrations of all CK metabolites. In the hypothesis of CK-equilibrium the theoretical velocity can be computed for each heart (see MATERIALS AND METHODS). Table 4 shows the comparison of the predicted and the NMR-measured velocity in the various protocols. For control hearts, the predicted flux was in good agreement with the NMR-measured flux (5.9 ± 0.2 and 6.5 ± 0.2 mM/s, respectively; n = 7 each). In all treated groups except H45, predicted flux was significantly higher than measured flux. Neither the decreased CK flux in severe hypoxia nor the constant flux with cyanide perfusion can be predicted from the theoretical velocity calculated from the hypothesis of CK equilibrium. Several hypotheses could explain this discrepancy: CK activity is modulated by factors other than the concentration of its metabolites; the concentrations of these metabolites are not the actual concentr-
tions effective at the site(s) of CK; or the complexity of compartments in the cell cannot be accounted for in a simplified equilibrium hypothesis of all CK isoforms.

Relation between cardiac performance and CK and ATP synthase fluxes. Figure 6A shows the relation between CK flux and RPP for hypoxic and cyanide-treated hearts. In cyanide a threefold decrease in systolic function occurred without any change in CK flux. Similarly, in mild hypoxia, CK flux remained constant for a twofold reduction in RPP. Only for severe hypoxia could the progressive decrease in cardiac performance (expressed as RPP) be related to impaired CK flux [for hypoxia (PO$_2$ $\leq$ 45%), a linear relation could be observed with a regression coefficient ($r^2$) $= 0.63$ calculated from individual hearts]. In this latter case, CK flux decrease was also associated with a progressive rise in EDP as hypoxia became more severe (Fig. 6B).

The $Q\dot{O}_2$ and the ATP synthesis rate can be calculated for each NMR-perfused heart from its RPP (see MATERIALS AND METHODS) (Table 5). In all cases, CK flux remained at least threefold higher than ATP synthesis, suggesting that energy transfer by CK was never limiting.

$O_2$ supply to the hypoxic heart. To understand the striking differences in the pathway of CK energy transfer of hearts submitted to mild inhibition of ATP synthesis (CN and H$_{45}$) or severe hypoxia (H$_{35}$ and H$_{25}$), we tested the hypothesis that severe hypoxia induced a drastic shortage in $O_2$ supply. $Q\dot{O}_2$ and cardiac performance were analyzed in relation to Pa $O_2$ (inflow) and Pv $O_2$ (effluent) at various levels of hypoxia. The relation between $PvO_2$ and Pa $O_2$ is shown in Fig. 7A: $PvO_2$ decreased linearly with aortic PO$_2$ down to $\leq 40\%$ of the control Pa $O_2$ (206 mmHg), and $PvO_2$ was $< 20$ mmHg for Fig. 3. CK flux during inhibition of ATP synthesis. A: $k_f$ in control, hypoxia [45 (H$_{45}$), 35 (H$_{35}$), 25 (H$_{25}$) % of control $O_2$ in perfusate], and cyanide perfusion [for various cyanide concentrations (CN$_I$, 0.15; CN$_{II}$, 0.20; CN$_{III}$, 0.25; CN$_{IV}$, 0.25 mM) with pacing groups]. B: CK forward flux ($k_f$·[PCr], where [PCr] is PCr concentration) in various groups. Significantly different from control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

![Graph A](image1)

![Graph B](image2)

Fig. 4. CK flux measured in individual hearts. A: paired analysis of CK flux at various level of hypoxia confirmed constancy of CK flux for mild hypoxia and its progressive decrease in severe $O_2$ restriction. Each symbol corresponds to an individual heart (n = 8). Pa$_2$, "arterial" PO$_2$ measured just above aorta. B: CK flux was never affected by cyanide (n = 5 from groups CN$_I$ and CN$_{III}$).

![Graph C](image3)

Fig. 5. Relation between CK flux and PCr content in control, hypoxia, and cyanide-perfused hearts. CK flux appears linearly related to PCr in hypoxia ($y = 0.59x - 2.2$, $r^2 = 0.82$, with $y$ = CK flux in mM/s and $x$ = PCr in mM) but independent of PCr with cyanide perfusion ($r^2 = 0.02$).
more severe hypoxic perfusion. Below this threshold, myocardial O2 consumption (in μmol O2 ·g wet wt −1·min −1); CK/ATP synth, ratio of flux of CK and ATP synthesis. ATP synthesis rate (expressed in mM/s) is estimated from Q˙O2 (see MATERIALS AND METHODS) and corresponds to a maximal estimate of ATP synthesis rate. Significantly different from control: a P < 0.05; b P < 0.01; c P < 0.001. Comparison between CN groups and H25 group: d P < 0.05; e P < 0.01; f P < 0.001.

**DISCUSSION**

Our aim was to reevaluate the relation between energy transfer by CK and energy supply and demand in the isolated, perfused heart during impairment of ATP synthesis. Graded inhibition, achieved by decreasing the O2 content of the perfusate or by low concentrations of cyanide, was designed to induce similar impairments in energetic demand. Our main observation was the absence of a relation between heart function and CK forward flux measured by saturation-transfer NMR during moderate inhibition of ATP synthesis: CK flux remained constant despite a two- to threefold decrease in mechanical performance and Q˙O2. On the other hand, severe hypoxia induced a parallel decrease in both performance and CK flux; we discuss here the origin of this impaired CK flux.

**Table 4. Comparison of measured and predicted velocity of creatine kinase forward reaction**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Vmeas</th>
<th>Vtheor</th>
<th>Difference Vtheor − Vmeas</th>
<th>%Free Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>6.5 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>−0.1 ± 0.6</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>H45</td>
<td>4</td>
<td>5.3 ± 0.5</td>
<td>6.9 ± 0.3</td>
<td>1.4 ± 0.6</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>H35</td>
<td>3</td>
<td>3.1 ± 0.5</td>
<td>7.5 ± 0.4</td>
<td>4.4 ± 0.2</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>H25</td>
<td>8</td>
<td>1.9 ± 0.2</td>
<td>8.0 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>CN1</td>
<td>5</td>
<td>6.0 ± 0.4</td>
<td>8.4 ± 0.3</td>
<td>2.4 ± 0.7</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td>CN11</td>
<td>3</td>
<td>6.4 ± 0.6</td>
<td>8.7 ± 0.2</td>
<td>2.3 ± 1.2</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>CN111</td>
<td>6</td>
<td>5.7 ± 0.3</td>
<td>9.0 ± 0.4</td>
<td>3.3 ± 0.6</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>CN11V</td>
<td>3</td>
<td>5.8 ± 0.5</td>
<td>8.6 ± 0.4</td>
<td>2.8 ± 0.9</td>
<td>6.0 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. Measured (Vmeas) and predicted (Vtheor) velocities are expressed in millimolar per second, free enzyme in % of total. In all treated groups except H45, flux predicted from metabolite concentrations in each heart was higher than measured as shown by the difference Vtheor − Vmeas. Significantly different from control: * P < 0.05; † P < 0.01; ‡ P < 0.001.

**Fig. 6. Relation between CK flux and cardiac performance.** A: CK flux as function of rate-pressure product. CK flux was constant in cyanide groups despite threefold decrease in systolic activity. Similar contractile dysfunction induced by severe hypoxia (<40% of control O2) results in CK flux impairment. Moderate hypoxia (H45) does not affect CK flux. B: CK flux as function of end-diastolic pressure (EDP). Rise in EDP occurring in cyanide groups is modest compared with huge contracture developing together with impaired CK flux in severe hypoxia.

**Table 5. Creatine kinase and ATP synthesis flux**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Q˙O2</th>
<th>ATP Synthesis</th>
<th>CK/ATP synth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>7.9 ± 0.6</td>
<td>1.82 ± 0.13</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>H45</td>
<td>4</td>
<td>4.8 ± 0.5</td>
<td>1.10 ± 0.10</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>H35</td>
<td>3</td>
<td>3.9 ± 0.5</td>
<td>0.90 ± 0.12</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>H25</td>
<td>8</td>
<td>2.9 ± 0.3</td>
<td>0.66 ± 0.07</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>CN1</td>
<td>5</td>
<td>5.5 ± 0.2</td>
<td>1.25 ± 0.05</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>CN11</td>
<td>3</td>
<td>3.5 ± 0.2</td>
<td>0.81 ± 0.05</td>
<td>8.2 ± 1.9</td>
</tr>
<tr>
<td>CN111</td>
<td>6</td>
<td>3.7 ± 0.2</td>
<td>0.86 ± 0.04</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>CN11V</td>
<td>3</td>
<td>4.2 ± 0.3</td>
<td>0.97 ± 0.07</td>
<td>5.9 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. Q˙O2, measured O2 consumption (in μmol O2 ·g wet wt −1·min −1); CK/ATP synth, ratio of flux of CK and ATP synthesis. ATP synthesis rate (expressed in mM/s) is estimated from Q˙O2 (see MATERIALS AND METHODS) and corresponds to a maximal estimate of ATP synthesis rate. Significantly different from control: *P < 0.05; †P < 0.01; ‡P < 0.001. Comparison between CN groups and H25 group: a P < 0.05; b P < 0.01; c P < 0.001.

Constant CK flux measured by saturation transfer during moderate inhibition of ATP synthesis. CK kinetics obviously depend on the concentration of both substrates and products. In vitro, large changes in concentration are required to induce changes in the flux of the enzyme; for example the Km of MM-CK for PCR is ~2 mM (12). Similarly in the perfused heart, using our normoxic model of adenylate depletion by 2-DG (9), we demonstrated that CK flux was in a large range independent of ATP and PCR concentration (23). Indeed, ATP and PCR must reach concentrations of ~4 mM to start to impair CK flux in this normoxic model. Here, partial inhibition of ATP synthesis resulted in minimal concentration of 5.3 and 4.5 mM for PCR and ATP, respectively (Table 2). Thus, in all our inhibitory conditions, including severe hypoxia, the mean concentrations of PCR and ATP did not reach the range necessary to markedly affect the kinetics of cytosolic CK flux. In the hypothesis of CK equilibrium, and without taking into account CK compartmentation, a constant CK flux is thus expected from a kinetic point of view.
Independence of CK flux and systolic performance during moderate inhibition of ATP synthesis. In a normoxic myocardium, an increase in workload associated with rapid changes in the rates of ATP synthesis occurs without major changes in intracellular levels of ATP and PCr (for review, see Ref. 3); conversely, systolic activity is largely independent of the cytosolic concentrations of CK metabolites (9). To understand the continuous balance between ATP synthesis and utilization, two types of mechanisms have been suggested. The first mechanism is a parallel activation of ATPases and ATP synthesis pathways by calcium, and the other mechanism involves signaling between both sites by metabolites of CK. In this latter hypothesis, one expects a correlation between the rates of ATP synthesis by oxidative phosphorylation, the rate of energy transfer by CK, and the rates of ATP utilization by ATPases. CK flux is thus assumed to be related to ATP synthesis and utilization (6). This is presumed to be a specificity of the myocardium caused by the high proportion of bound CK isozymes (~50% of total CK). Indeed, in skeletal muscle, the 10-fold increase in ATPase rate observed between rest and maximal activity occurs without any modification of CK flux. However, this is also observed in the myocardium. In vivo, CK flux remains constant during a threefold increase in work induced by pacing or catecholamine stress (11, 13, 19). Conversely, in the perfused heart, a direct 99% inhibition of CK activity by iodoacetamide (IAAM) does not alter the baseline systolic parameters, although this nearly abolishes the contractile reserve (26). Moreover, a transition from low to high work (RPP from 1.5 to $4.5 \times 10^4$ mmHg·beat·min$^{-1}$) doubles ATP synthesis but only moderately affects CK flux (6). Only during the transition from arrest by KCl to a low systolic activity was a parallel threefold increase in ATP synthesis and CK flux observed (6), although such a huge change is not consistently detected (12, 14). More recently, the KCl-arrested heart has been recognized to be a puzzling situation in which the set of kinetic constants that predicts CK flux in a beating heart cannot be used (15). Thus, apart from this specific case, the evidence of a relation between work and CK flux appears rather weak in the normoxic myocardium both in vivo and in the perfused heart. Our results with cyanide and moderate hypoxia agree with this theory: a threefold change in ATP synthesis and utilization pathway occurs without any change in the flux of energy transfer by CK as measured by NMR saturation transfer.

CK flux and diastolic properties. In severe hypoxia, the decrease in CK flux was associated with a marked rise in EDP (Fig. 6B). We favor the hypothesis that this is caused by the formation of strongly attached cross bridges (rigor type contracture); low CK activity impairing ADP rephosphorylation that would result in an inhibition of myosin ATPase. In Triton-skinned fibers, such rigor force is evidenced when MgATP concentration is not sufficient to allow the detachment of myosin from actin; half-maximum rigor force occurs for an apparent concentration of MgATP of $300 \mu M$ in the absence of CK and $10 \mu M$ when MM-bound CK is functional and locally rephosphorylates the ADP produced by the ATPase (for review, see Ref. 29). Even in severe hypoxia, the cytosolic ATP concentration (~4 mM), which was at least one order of magnitude higher than the apparent $K_m$ of the ATPase for MgATP, should not be responsible for a marked rise in EDP. On the other hand, the cytosolic free ADP concentration (30–120 µM) is in the range likely to influence the ATPase and to induce rigor development (29). Indeed, in the perfused heart, increase in free ADP induced by a direct inhibition of CK by IAAM causes a dose-dependent increase in EDP that has been demonstrated to be caused by rigor (25). A rise in EDP was observed here in all conditions of inhibition of respiration. However, at variance with the IAAM model, no general relation could be observed between the free ADP calculated...
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from the CK equilibrium and the rise in EDP. With cyanide, the free ADP concentration was two times higher than in severe hypoxia, but the rise in EDP was modest, three times lower than in H316 (Tables 1 and 2). This is compatible with the fact that in skinned fibers rigor is prevented, even in the presence of 20 mM ADP in the bulk solution, if MM-bound CK is functional (28). This suggests that in cyanide the high CK activity is sufficient to rephosphorylate the ADP in the vicinity of ATPases and prevent their inhibition despite the high cytosolic free ADP as calculated using the equilibrium constant. By contrast, the impaired CK flux in severe hypoxia could not prevent a local rise in ADP and subsequent myofibrillar ATPase inhibition. This points again to the importance of bound CK for the myocardi- 
dium and further suggests that a rise in calculated cytosolic free ADP does not automatically reflect change in ADP at the sites of interest in the subcompartments.

Impaired CK flux in severe hypoxia: hypothesis of myocardial heterogeneity. The decreased CK flux ob-
erved for severe hypoxia is in agreement with previous reports both in the isolated heart perfused with 20% O2 (8) and, in vivo, in the open-chest rat respiring mix-
tures with O2 reduced from 21 to 8% (5). As discussed in Constant CK flux during moderate inhibition of ATP synthesis, the concentration of CK metabolites induced by severe hypoxia is not expected to decrease CK flux, as confirmed by the calculation of the theoretical CK flux (Table 4). Because CK velocity also depends on the concentration of active enzyme (i.e., saturated with substrate) an increasing proportion of free enzyme would decrease CK flux. This was not the case, because the increase in free enzyme did not reach significance. Alternatively, as suggested in longer-lasting ischemia, a loss of CK molecules or their inactivation by free radicals could be responsible for the impaired hypoxic flux. We do not favor these hypothesis because both the leakage of cytoplasmic enzymes (CK and lactate dehydrogenase) and the inactivation of CK occur only during the phase of reoxygenation (4).

In our model, in the absence of glucose, the O2 content of the cardiac effluent became negligible and the heart function was severely impaired when the PO2 in the perfusate was decreased below 40% of control (~200 mmHg, Fig. 7). As a result of myocardial cell PO2, marked O2 gradients are well known to occur along the path- 
way of capillaries. Even at the level of one cell, increasing PO2 results in a radial gradient of O2 whereas cyanide inhibition does not induce such heterogeneity (24). At the organ level, decreasing O2 supply below a “critical PO2” results in a patchwork of areas with different metabolic characteristics (for review, see Ref. 18). Obviously, this critical PO2 depends on the working conditions and the type of substrate available. Indeed, in the isolated heart using glucose as substrate, this critical PO2 is lower (data not shown). Such would also be the case in vivo because mixed substrates are available. Thus we suggest that in mild hypoxia all cells receive adequate O2 supply and/or are able to downregulate their ATP utilization and ATP synthesis (7). As hypoxia becomes more severe, an increasing number of cells do not receive adequate O2 supply, stop developing systolic activity, and enter the state of rigor. In these anoxic cells, one (or several) of the CK metabolites may reach concentrations inhibiting CK. The linear relation existing between impaired mechanical performance and CK flux in severe hypoxic hearts (in the range of 40 to 20% O2) would thus result from a progressive increase in the proportion of CK-inhibited cells. Such a correlation is also consistently observed, in the same range of cardiac performances as found here, in another model of tissular heterogeneity, reper-
fusion after long-term ischemia (17). Interestingly, a decrease in CK flux is also observed in vivo when rats inspire air with reduced O2 content (5). This suggests that the impairment of CK flux in severe hypoxia is not the consequence of the known limitation in oxygenation of the crystalline solution-perfused heart but reflects a more common feature linked to the imbalance in O2 supply and demand.

Considerations on NMR-measured CK flux and its physiological interpretation. The absence of modifica-
tion of CK flux observed here for a threefold change in heart function could be directly discussed, as previ-
ously suggested, as evidence for CK functioning at equilibrium everywhere in the cell. However, this obser-
vation is difficult to reconcile with the abundant litera-
ture describing the function of the CK isozymes in myofibrillar and mitochondria subcellular prepara-
tions (2, 20–22, 28–31).

NMR is generally assumed to reflect all unidirec-
tional CK fluxes originating from the cytosolic and the bound isoenzymes. Indeed, mito-CK flux can be de-
tected in isolated mitochondria (10) and in transgenic mice in which the homodimeric MM-CK gene has been knocked out (27). The NMR-detected forward CK flux thus represents the contribution of forward fluxes of cytosol, myofibrils, and mitochondria. For various working conditions, the total forward CK flux would be expected to be constant if all cytosolic and bound CKs are at equilibrium but also if opposite changes occurred in the forward mitochondrial and myofibrillar flux. Such precise tuning of both myofibrillar and mitochon-
drial CK activity is indeed a prerequisite of the continu-
ous balance of energy production and utilization in the hypothesis of the PCr-Cr shuttle and of the intracellu-
lar signaling role of CK metabolites. This aspect needs careful reevaluation before any definitive conclusion can be drawn from the NMR data in terms of physiologi-
ical importance of the CK in the whole organ.

In conclusion, during inhibition of ATP synthesis, heart function is not related to NMR-measured CK flux except during severe hypoxia. Our results suggest that the progressive impairment of CK flux in severe hypoxia results from an increasing number of cells with nonfunctional CK, a situation also likely to occur in pathological cases such as infarct or ischemic insult.

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