Functional Development of the Creatine Kinase System in Perinatal Rabbit Heart

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The functional development of the creatine kinase system has been studied in rabbit heart during perinatal growth. Fiber bundles were obtained from left ventricles of fetal rabbits at the 30th day of gestation, newborn rabbits aged 1, 3, 8, and 17 days, and adult rabbits. Total creatine kinase activity was constant during perinatal development, whereas myofibrillar bound creatine kinase activity increased 15-fold during the first postnatal week. Functional activity of myofibrillar creatine kinase was assayed in Triton X-100-skinned fibers by its ability to induce active tension in the absence of ATP or to relax rigor tension. It was very low in 1-day-old newborns and increased during the first 2 weeks to reach adult levels 17 days after birth. Functional activity of mitochondrial creatine kinase was determined in saponin-skinned fibers. Creatine-stimulated respiration appeared only after birth and increased gradually between 1 and 17 days after birth. The results show that, although the two creatine kinase isozymes (mitochondrial and myofibrillar) are expressed at different stages during development, their functional activities appear in parallel in mitochondria and myofibrils. Early postnatal development is characterized by binding of creatine kinase isoenzymes to intracellular organelles. Such compartmentation participates in the postnatal cardiac cellular maturation. (Circulation Research 1991;69:665–676)

Creatine kinase (CK) is an important enzyme in the energy metabolism of muscle cells, catalyzing the reversible transfer of a phosphate moiety between ATP and creatine without loss of free energy. CK exists as a dimer composed of two subunit types, M and B, giving three isoenzymes, MM, MB, and BB. In addition, there is a fourth CK isoenzyme in the mitochondria, Mt-CK, which differs biochemically and immunochemically from cytosolic forms. Studies with subcellular fractionation or histochemical localization have revealed that CK isoenzymes are present in cytosol or bound to intracellular structures at the sites of energy production and energy utilization, such as plasma membranes, sarcoplasmic reticulum, nuclei, myofibrils, and mitochondria.

In the myofibrils, CK has been identified as the MM-type isoenzyme and was shown to be localized in the middle of the A band (i.e., the M line) as well as on the entire thick filament. MM-CK is found near the location of ATPase activity and has been shown to be biochemically and functionally coupled to myosin ATPase.

In cardiac muscle, the activity of MM-CK can rephosphorylate virtually all the intramyofibrillar ADP generated during maximal myosin ATPase activity and contraction. Furthermore, myosin ATPase reaction preferentially uses ATP supplied by the CK reaction rather than cytosolic ATP.

In mitochondria, Mt-CK is present at the outer surface of the inner membrane. The high efficiency of phosphocreatine (PCr) formation in isolated heart mitochondria under conditions of oxidative phosphorylation was demonstrated by Jacobus and Lehninger. The increased rate of PCr production from mitochondrial ATP has been explained by CK coupling to the ATP/ADP translocase, which was suggested to supply ATP directly to CK. This process removes ADP and thus drives the CK reaction in the reverse direction of PCr synthesis. In cardiac cells, it has been proposed that, in addition to the role of energy reservoir for contraction, the various locations of the CK isoenzymes permit microcompartmentation and functional coupling near the sites of energy production and consumption.

Fetal development is accompanied by a sharp increase in total CK activity. The relative content of the different CK isoenzymes varies during perinatal
development. The major isoform present during early fetal life is the BB isoform. Fetal differentiation and maturation of muscle cell is characterized by a decrease in the BB isoform and an increase in the specific muscular form of CK, MM-CK, in skeletal muscle\(^1\) as well as in the cardiac cell.\(^1\) The synthesis of MM-CK always precedes that of Mt-CK. In heart, this isoform appears at the end of the fetal life in precocious animals or during postnatal development.\(^1\) Thus, the two main isoforms that are specifically linked to the sites of production (Mt-CK) and the sites of utilization (MM-CK) appear at different times during development. However, the presence of the specific isoforms of CK does not tell about their localization and function in intracellular structures. For example, cytosolic MM-CK is present during fetal life in rat heart, but maturation of the M line and the presence of CK in myofibrils are detected only after birth.\(^2,3\)

This study was undertaken to follow the appearance of functional activity of the two main CK isoforms in myofibrils and mitochondria during late fetal life and early postnatal development using skinned fibers. The results show that although the main CK isoforms in cardiac tissue are expressed at different stages during perinatal development, their functional activities appear in parallel in mitochondria and myofibrils. This result reinforces the hypothesis of a role of compartmentation of energy in adult cardiac cells and provides evidence that the CK system is part of the complex organization of the highly differentiated mammalian cardiac cell.

**Materials and Methods**

**Animals**

New Zealand rabbits obtained from the Institut National de la Recherche Agronomique (Centre National de la Recherche Zooloigique [CNRZ], Jouyen Josas, France) were used for this study. All experimental animals were anesthetized with intravenous or intraperitoneal pentobarbital sodium according to the recommendations of the Institutional Animal Care Committee, INSERM, Paris, and hearts were rapidly removed. The rabbits were studied in a random order. Fibers were obtained from the left ventricle of fetal rabbits at the 30th day of gestation (24 hours before birth), 1-day-old newborn rabbits (within 24 hours after birth), immature (3-, 8-, and 17-day-old) rabbits, and young (8–11-month-old) adult rabbits. At least three different rabbits in each group were used. The same rabbits were used for biochemical and mechanical experiments.

**Mechanical Experiments**

**Fiber preparation.** Triton X-100 treatment induces complete disruption and vesiculization of all cellular membranes, resulting in removal of the cytosolic and membrane-associated fractions of proteins.\(^1\)

Muscle fiber bundles were dissected from left ventricular papillary muscles and tied at both ends with a natural silk thread in a zero-calcium Krebs’ solution, pH 7.4. Fibers were incubated for 1 hour in a relaxing solution (pCa 9, see solutions below) containing 1% Triton X-100 to solubilize the membranes and were then transferred to the relaxing solution without detergent. After the skimming procedure, the fiber was mounted in the experimental setup, adjusted to slack length, and stretched by 20% of slack length. It was then stimulated in the activating solution (pCa 4.5, see below) and made to relax in the relaxing solution. Fiber length was rechecked and, if necessary, readjusted. The length and diameter of the muscles were measured using a graticule in the dissecting microscope.

**Experimental apparatus.** Force and length changes were measured as described previously.\(^2,2\) The fibers were mounted between two stainless-steel hooks. One hook was connected to a transducer (model AE 801, Aker’s Microelectronics, Horten, Norway). The band width of the transducer and hook was 2 kHz. The other hook was connected to the coil of a standard loudspeaker (model TS-130A, Pioneer Electric & Research Corp., Forest Park, Ill.). The coil was glued to a glass tube axis (2 mm in diameter) driven in an axial ball bearing. This gave a total moving mass of <1.5 g. A flag with a narrow window was glued on the glass axis between a lamp and a position detector (type S1543, Hamamatsu, Japan), allowing measurements of the displacement length. A feedback with the length signal combined with a power amplifier allowed control of muscle length. The system had a rise time of about 1 msec without overshoot. Length and force changes were monitored on a digital storage oscilloscope (model OS4020, Gould, Inc., Cleveland, Ohio). Force traces were digitized at 20 kHz (12-bit analog/digital converter) and analyzed on-line using a computer (COMPAQ Deskpro 286, COMPAQ Computer Corp., Houston, Tex.) and stored on videotape.

Muscles were immersed in 2.5-ml chambers arranged around a disk and immersed in a temperature-controlled bath positioned on a magnetic stirrer. Each solution was well stirred at high speed (>1,000 rpm). All experiments were performed at 22°C.

**Solutions.** Relaxing (pCa 9, solution A) and activating (pCa 4.5, solution B) solutions were calculated as described previously.\(^1\) All solutions (Table 1) were calculated to contain (mM) EGTA 10, imidazole (pH 7.1) 30, Na\(^+\) 30.6, Mg\(^{2+}\) 3.16, and dithiothreitol 0.3; ionic strength was adjusted to 0.16 M with potassium acetate. Standard relaxing and activating solutions also contained 3.16 mM MgATP and 12 mM PCr. CK-relaxing (solution E) and CK-activating (solution F) solutions designed to check the CK efficacy contained 250 \(\mu\)M MgADP and 12 mM PCr with no ATP. Rigor solutions were obtained by mixing two solutions of pMgATP 6 and 2.5 at pCa 9 (solutions A4 and E in the presence of PCr, and solutions C and G in the absence of PCr).

EGTA was obtained from Sigma Chemical Co., St. Louis, Mo. PCr (Neoton, Schiapparelli Farmaceutica, Turin, Italy) was a kind gift of Prof. E. Strumia.
### Table 1. Composition of the Different Solutions Used for Mechanical Experiments

<table>
<thead>
<tr>
<th></th>
<th>With ATP</th>
<th></th>
<th>With ADP</th>
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<th>With PCr</th>
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<tr>
<td></td>
<td>Relaxing (A)</td>
<td>Activating (B)</td>
<td>CK-relaxing (E)</td>
<td>CK-activating (F)</td>
<td>Relaxing (A)</td>
<td>Rigor (E)</td>
<td>Relaxing (C)</td>
<td>Rigor (G)</td>
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<td>96</td>
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<td>Na⁺ (mM)</td>
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<tr>
<td>EGTA (mM)</td>
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<td>DTT (mM)</td>
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</tbody>
</table>

*pCr, phosphocreatine; CK, creatine kinase; DTT, dithiothreitol. pH = 7.1 for all solutions. See text for details.*

**Experimental protocol.** Quick length changes, 0.3–3% of initial muscle length, were applied in the relaxing and activating solutions. Transient tension responses were digitized by a DT2801 AD card (Data Translation) and analyzed on-line. A series of 12 stretches and releases was successively applied first in relaxing solution A, then twice in activating solution B, then in CK-activating solution F, and then in rigor solution G without substrates. Each reported value is the mean of five to seven determinations after stretches of varying amplitudes in a given experimental condition.

Stiffness was the extreme tension reached during stretch (mN·mm⁻²) divided by the length change (μm). Passive stiffness was measured in the relaxing solution at the beginning of each experiment. Stiffness (linked to actively cycling crossbridges) was calculated as the difference between total stiffness measured in the activating solution minus passive stiffness. Indeed, at low calcium and high ionic strength, no myosin heads are found associated with the thin filament in cardiac skinned fibers; passive stiffness thus reflects elastic elements in parallel. Active stiffness was also normalized to maximal force obtained at the beginning of the experiment in the activating solution to cancel tension variations due to both creep and fluctuations from one measurement to another.

Rigor tension relaxation experiments were carried out by bathing the fibers in low calcium solutions (pCa 9) containing decreasing amounts of MgATP in the presence or the absence of PCr. Data were fitted using the Hill equation: T = (MgATP)ᵣ₀/[K⁺ + (MgATP)ᵣ₀], where T is relative tension, n₉₀ is the Hill coefficient, and K is the dissociation constant. The pMgATP for half maximal relaxation (pMgATP₉₀ = -log₁₀(K/n₉₀)) was calculated for each experiment using linear regression analysis.

**Mitochondrial Respiration Experiments**

Fiber preparation. For determination of mitochondrial respiratory parameters in saponin-skinned fibers, we used the method described previously, with minor changes. The selective effect of saponin could be explained by its affinity for cholesterol, which is higher in content in sarcolemma than in mitochondria and sarcoplasmic reticulum; thus, the ultrastructure of mitochondria and sarcoplasmic reticulum remains intact. Conditions of the treatment and composition of the incubation medium used made it possible to obtain skinned fibers with functionally intact mitochondria.

Briefly, fiber bundles, 200–300 μm in diameter, were isolated from the endocardial surface of the left ventricle and transferred into solution with EGTA (solution S, see below). Bundles were incubated with intense shaking for 30 minutes in solution S containing 50 μg/ml saponin. Bundles were then washed for 10 minutes in solution R (see below) without high-energy phosphates. All the procedures were carried out at 4°C.

Solutions S and R contained (mM) EGTA 10 (pCa 7), free Mg²⁺ 3, taurine 20, dithiothreitol 0.5, and imidazole 20 (pH 7.0). Ionic strength was adjusted to 0.16 M by addition of potassium 2-(N-morpholino)ethanesulfonate. Solution S also contained 5 mM MgATP and 15 mM PCr. In place of high-energy phosphates, solution R contained 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/ml fatty acid–free bovine serum albumin.

**Measurement of respiratory parameters.** The respiratory rates were determined by a Clark electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) in an oxygraph cell containing 10–15 bundles in 3 ml solution R at 22°C with continuous stirring. The solubility of oxygen at 22°C was taken to be 460 ng atoms/ml. Bundles were introduced into the oxygraph cell, and various respiratory parameters were measured by sequential substrate addition. The parameters measured were as follows: basal respiration after addition of 5 mM glutamate and 2 mM malate, respiration rate after addition of 0.1 mM ADP, creatine-stimulated respiration after addition of 20 mM Cr, maximal respiration rate after addition of 1
mM ADP, and respiration rate after addition of 35 μM carboxyatractyloside (an inhibitor of ATP-ADP translocase). Endocardial fibers were then dried and weighed. Respiration rates were expressed as ng atoms of oxygen/min/mg dry wt.

Biochemical Determinations

Part of skinned fiber bundles prepared from left papillary muscles for mechanical experiments were used to measure myofibrillar ATPase and CK activities.

All solutions of the same basic composition as for mechanical experiments contained (mM) EGTA 10, imidazole 30 (pH 7.1), Na+ 30.6, Mg2+ 3.16, and dithiothreitol 0.3; ionic strength was adjusted to 0.16 M with potassium acetate.

ATPase activity was measured directly from skinned fibers attached to a hook in the fluorometer at maximal calcium activation (pCa 4.5). ATPase was assessed by the release of ADP coupled to pyruvate kinase and lactate dehydrogenase at pCa 4.5 in the presence of 0.5 mM phospho(enol)pyruvate, 40 μM NADH, 2 IU/ml pyruvate kinase, and 1.5 IU/ml lactate dehydrogenase. The reaction was started by the addition of 3 mM ATP. Functional coupling of CK to ATPase was measured by the apparent decrease in ATPase activity on addition of 10 mM PCr. The ADP produced by ATPase is locally rephosphorylated by bound CK and thus becomes unavailable to the bulk pyruvate kinase.10

Myofibrillar CK was extracted by 90-minute incubation at room temperature in a solution containing (mM) Tris 5, EGTA 1, and dithiothreitol 1, at pH 8, vigorously stirred. CK activity as assayed fluorometrically by NADPH production in a reaction coupled to hexokinase and glucose-6-phosphate dehydrogenase (2 IU/ml each) at pCa 9 in the presence of (mM) ADP 0.5, NADP 0.8, glucose 20, and AMP 10 (to inhibit myokinase). After stabilization of the fluorescence level, the CK reaction was started by the addition of 10 mM PCr. In a few cases, CK activities were also measured in right ventricles of the same rabbit. The amount of myofibrillar proteins in the assay was similar for all ages and ranged from 10 to 60 µg protein (mean, 32±2; n=88).

Total CK activity was measured in the left ventricle after Ultra-Turrax homogenization in solution A. All enzymatic determinations were performed at pH 7.1 and 22°C. Protein content was measured by Lowry assay in the fibers and by biuret assay on ventricular homogenates.

Table 2. Heart Weight and Body Weight

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>HW (mg wet wt)</th>
<th>BW (g)</th>
<th>HW/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult (n=4)</td>
<td>7,903±456</td>
<td>3,600±89</td>
<td>2.19±0.08</td>
</tr>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 days (n=9)</td>
<td>1,027±37</td>
<td>292±17</td>
<td>3.54±0.10</td>
</tr>
<tr>
<td>8 days (n=3)</td>
<td>785±76</td>
<td>183±26</td>
<td>4.34±0.21</td>
</tr>
<tr>
<td>3 days (n=5)</td>
<td>313±24</td>
<td>58.6±4.2</td>
<td>5.36±0.27</td>
</tr>
<tr>
<td>1 day (n=6)</td>
<td>297±26</td>
<td>53.0±2.5</td>
<td>5.57±0.34</td>
</tr>
<tr>
<td>Fetal (n=9)</td>
<td>243.5±4.0</td>
<td>40.0±1.0</td>
<td>6.09±0.01</td>
</tr>
</tbody>
</table>

Values are mean±SEM. HW, heart weight; BW, body weight. All newborn and fetal values are significantly different from adult values (p≤0.001).

Results

Anatomic Data

Characteristics of the rabbits are shown in Table 2. During perinatal development, there is a progressive increase of heart weight and body weight as expected. However, the heart weight/body weight ratio considerably decreased from 6.09 mg heart wt/g body wt in the fetus to 2.19 mg heart wt/g body wt in the adult.

Myofibrillar Creatine Kinase Activity

Activities of total and myofibrillar bound CK were compared in late fetal, newborn, and adult rabbits. Total tissue CK activity per milligram total protein did not significantly change with age (Figure 1A). Myofibrillar CK was measured as CK activity present in fibers after skimming with 1% Triton X-100. Myofibrillar bound CK increased about 15-fold in the first postnatal week and remained similar thereafter (Figure 1B). Adult Triton X-100–skinned fibers contained only the MM isofrom as checked by agarose electrophoresis (result not shown).

Bound myofibrillar activity was not significantly different in the left and the right ventricle. For the left and right ventricle, respectively, values were (IU/mg protein) 0.21±0.03 and 0.22±0.02 (n=8) in 17-day-old newborns, 0.03±0.01 and 0.04±0.01 (n=4) in 3-day-old newborns, and 0.008±0.001 and 0.007±0.001 (n=6) in fetal rabbits. Although myofibrillar CK was not different between left and right ventricles, only left ventricular fibers were used in further experiments.

Thus, while total CK activity remained constant during development, myofibrillar CK activity increased with age, reaching adult values 8 days after birth.

The rephosphorylating activity of bound CK can be demonstrated by the inhibition of ADP release during ATPase assessment. This method involves competition between endogenous CK and exogenous pyruvate kinase for MgADP produced by the ATPase in the presence of high calcium (pCa 4.5). In fully activated skinned adult fibers, the addition of PCr activates endogenous CK to rephosphorylate MgADP in situ inside the myofibrils, and the re-
lease of MgADP in the medium practically stops. In Figure 2 shows evidence of such a coupling of CK to ATPase activity in the adult heart. Inhibition of endogenous CK by fluorodinitrobenzene (FDNB) completely reversed the effect (not shown). At birth, when myofibrillar CK activity was low, the addition of PCr weakly affected the ADP release (Figure 2). Inhibition of ADP release was expressed as percentage of ATPase activity: 28±4% (n=13) in fetal rabbits, 28±8% (n=17) in 1-day-old newborns, 49±3 (n=15) in 3-day-old newborns, 70±3 (n=11) in 8-day-old newborns, 78±6 (n=7) in 17-day-old newborns, and 89±2 (n=14) in adults. This supports an increased biochemical coupling between ATPase and myofibrillar CK with maturation.

**Functional Properties of Myofibrillar Creatine Kinase**

The functional properties of myofibrillar CK were assayed by its ability to induce active tension in the absence of ATP or to relax rigor tension.

**Creatine kinase and active tension.** It has been shown, using adult rat heart skinned fibers, that myofibrillar CK was able to maintain normal mechanical function in the absence of MgATP and in the presence of MgADP and PCr by efficient local rephosphorylation of MgADP. Furthermore, it was shown that CK was able to maintain normal active stiffness, but after its inhibition by FDNB, stiffness increased because of reduced local ATP concentration and rigor tension. Indeed, stiffness is higher in rigor than in active tension because of the presence of slowly cycling or noncycling bound crossbridges. To determine the ability of myofibrillar CK to sustain active tension in young animals, stiffness measurements were made in control conditions, in CK-activating solution, and in rigor conditions. Active tension was generated by exposing the fibers to high calcium solution either in the presence of both 12 mM PCr and 3.16 mM MgATP (activating solution B) or in the absence of MgATP but in the presence of 250 μM MgADP and 12 mM PCr (CK-activating solution F, in which all the MgATP used by the myofibrillar ATPase is regenerated from MgADP by bound CK) and in the absence of MgATP and PCr (rigor solution G).

Quick length changes of 0.3–3% were applied to the muscles at pCa 4.5. The maximal tensions reached during the stretch were obtained in solutions A, B, F, and G and plotted as a function of the length change after subtraction of the passive properties of the fibers. Figures 3A and 3B show typical curves obtained in fibers from an adult and a 1-day-old rabbit, respectively. The responses in relaxing solution reflected the passive elastic properties of the muscles. In the adult, stiffness curves in control solution and CK-activating solution coincided with each other and were different from the rigor conditions, whereas in the newborn, the mechanical properties in CK-activating solution were close to rigor conditions. This result shows that the rephosphorylation of ADP by CK is sufficient to sustain active tension in adult cardiac muscle, whereas it is not sufficient in neonatal rabbit heart. Stiffness values in control, CK-activating, and rigor solution are reported in Table 3 for the different ages. Stiffness values that were expressed per muscle cross-sectional area increased with age as expected from increased myofibrillar volume. In adult and 17-day-old rabbits, stiffness in CK-activating solution was similar to control, whereas it was significantly higher in newborns of <8 days and in fetal rabbits. Conversely, stiffness in CK-activating solution was not statistically different from rigor stiffness in young (1-day-old and fetal) rabbits. The percent increase in stiffness between control and CK-activated values was plotted as a function of age showing the increasing functional
ability of myofibrillar CK to sustain tension with age (Figure 3C).

Relaxation of rigor tension. The next series of experiments was undertaken to study the influence of bound CK on the relaxation of rigor tension. A stepwise decrease in MgATP in the absence of calcium and PCr leads to the appearance of rigor tension. The presence of PCr in the medium shifts the dependence of the rigor tension on MgATP concentration toward much lower MgATP concentrations. Figure 4 shows rigor tension as a function of MgATP concentration in the absence of PCr in the adult rabbit heart and in the presence of 12 mM PCr in 1-, 3-, 8-, and 17-day-old rabbit hearts. The pMgATP/tension relations were fitted using the Hill equation, and the mean values of the pMgATP so in the presence and in the absence of PCr for the different ages are reported in Table 4. In the absence of PCr, rigor tension developed for similar MgATP concentrations at all ages. The addition of PCr shifted the sensitivity to MgATP toward lower concentrations in the adult, as already reported for other mammalian species. This shift progressively increased with age from newborn to adult. Since this shift is due to local ADP rephosphorylation by bound CK, it is possible to take its amplitude as an index reflecting the functional state of bound CK. In 17-day-old rabbits, the CK efficacy was the same as in adult rabbits, whereas it was significantly lower for younger rabbits. It can be seen from Figure 4 (see also Figure 7) that myofibrillar CK efficacy was very low in young rabbits and increased in the first 2 postnatal weeks to reach adult levels 17 days after birth. This probably reflects the binding of CK to myofibrils.

Functional Activity of Mitochondrial Creatine Kinase

To study the appearance of the functional activity of CK in mitochondria during development, mitochondrial function was determined in saponin-skinned fibers. This method is based on the fact that saponin perforates the sarcolemma because of a high amount of cholesterol, while intracellular membrane structures are still intact. It allows study of the total mitochondrial population in its environment without isolation of these organelles.

Figure 5 shows typical oxygraph recordings from saponin-skinned fibers of adult and newborn rabbits. The basal respiration rate in the presence of glutamate and malate was accelerated by addition of 0.1 mM ADP. Subsequent addition of 20 mM creatine, which activates mitochondrial CK and increases the ADP concentration in the vicinity of the inner mitochondrial membrane, enhanced respiration rate more in the adult than in the newborn. This enhancement indicates the functional efficacy of Mt-CK. The maximal rate of respiration was observed with 1 mM ADP, whereas addition of carboxyatractyloside, an inhibitor of ATP-ADP translocase, considerably depressed the respiration rate. Figure 6 shows that there were only slight alterations in the basal and maximal respiration rates per milligram of dry weight from late fetal to adult rabbits. However, after addition of creatine, the respiration rate was increased in adult but not in fetal and newborn fibers.

The values of the relative parameters of respiration that do not depend on the amount of mitochondria are listed in Table 5 for the different ages. The ratio of maximal rate to basal rate can be taken as an
Figure 3. Graphs showing stiffness measurements during rabbit left ventricular development. Stiffness is measured by the immediate force changes after quick length changes of varying amplitudes. Force changes were plotted as a function of length changes. Passive stiffness (○) is obtained in low calcium solution (pCa 9). Active stiffness (●) is obtained in the presence of 12 mM phosphocreatine and 3.16 mM MgATP at pCa 4.5. Creatine kinase (CK)–activated stiffness (■) is obtained in a solution containing 12 mM phosphocreatine and 250 μM MgADP at pCa 4.5. Rigor stiffness (▲) is obtained in the absence of ATP and phosphocreatine. Panel A: Adult: Fiber diameter, 240 μm; maximal tension, 30.7 mN/mm². Panel B: One-day-old newborn: Fiber diameter, 240 μm; maximal force, 17.8 mN/mm². Panel C: The difference between CK-activated and active stiffness relative to active stiffness plotted as a function of age. Value above bar indicates the number of experiments.

The analog of the respiration control index indicating the coupling between respiration and oxidative phosphorylation processes. No major changes were observed during development. A slightly lower value at 3 days was obtained, although absolute values for basal and maximal respiration were not different from adult values. The percentage of inhibition of respiration by carboxymethylcellulose, an index of the integrity of the mitochondrial inner membrane, was not different between groups. Thus saponin treatment did not seem to differentially affect the mitochondrial membrane.

The relative extent of stimulation of respiration by creatine reflects the modulation of respiration by the CK reaction. Creatine-stimulated respiration underwent dramatic changes during development. It appeared immediately after birth and increased gradually to reach adult value 17 days after birth (Table 5).

The functional parameters of mitochondrial and myofibrillar CK activities are plotted as the percentage of their values in adult rabbit heart as a function of age (Figure 7). This figure shows that myofibrillar and mitochondrial CK function appeared in parallel in developing rabbit heart during the first 2 weeks after birth.

Discussion

We have followed during fetal life and the early postnatal period the appearance of CK function in myofibrils and mitochondria of the rabbit heart. Indeed, by using skinned fibers to control intracellular medium it is possible to demonstrate the presence and function of CK associated with intracellular structures. In myofibrils the ATP-regenerating potential of bound CK was assessed by the ability of myofibrillar CK to maintain normal stiffness and to relax rigor tension in Triton X-100–skinned fibers. In mitochondria, the activity of mitochondrial CK was assessed by the ability of creatine to stimulate mitochondrial oxygen consumption in saponin-skinned fibers. The results show that, although the two isoforms associated with myofibrils and mitochondria are expressed at different stages of developing rabbit heart, their activities appear in parallel in these two organelles.

Birth is associated with the combination of increased circulating PO₂ and changes in peripheral vascular resistance, which increases the work load imposed on the left ventricle. Moreover, the remarkable decrease in the heart weight/body weight ratio in the first postnatal weeks suggests increased myocardial efficiency. The myocardium undergoes a shift from glycolytic to oxidative metabolism and major changes in contractility. Such an adaptation involves both morphological and enzymatic complex modifications.

The mammalian cardiac cell undergoes a rapid transition from partially anaerobic metabolism in the fetus to predominantly aerobic metabolism after birth. Mitochondrial volume, surface density of cristae, respiratory membrane area per unit myo-
fibrillar volume, cytochrome content, and ability to synthesize and oxidize long chain fatty acids markedly increased during the first postnatal week in rabbit hearts.

Selective perforation of sarcolemma by saponin allows skinned cardiac fibers to be obtained with morphologically and functionally intact mitochondria. In view of the marked increase in oxidative energy production associated with birth in vivo, it was at first surprising to observe that the maximal respiratory activity per milligram of fiber dry weight in vitro was similar in all age groups. This was neither due to partial uncoupling of fetal and newborn heart mitochondria, since good indexes of oxidative phosphorylation (acceptor control ratio between 6 and 8), were obtained for all age groups, nor was it due to fragility of the mitochondrial membrane, since the percentage of inhibition of respiration by carboxyatractyloside was not different (Table 5). In the rabbits as well as in other species, state III respiration per milligram mitochondrial protein has been shown to be greater in the perinatal period than in the adult. At birth, a combination of an increase in the amount of mitochondrial protein per total protein with a decrease in maximal respiratory capacity of mitochondria probably could account for the unchanged maximal respiration observed in our conditions of maximal oxygen and substrate availability.

At intermediate respiratory rates, closer to physiological conditions, respiration was stimulated by creatine in the adult rabbit heart but not in the fetal rabbit heart. After birth, a marked increased in creatine-stimulated respiration occurs, reaching the adult values by 17 days. Our results agree with the postnatal appearance of Mt-CK and the increase in its enzymatic activity between 3 and 18 days of age observed in the rabbit heart. Considerable species variation exists in Mt-CK development. Mt-CK appears before birth in precocious animals like the lamb and after birth in the mouse and rabbit, whereas in the rat heart, its specific activity still increases from weaning to adulthood. This suggests that the increase in PCr per se is not a main determinant of Mt-CK expression. It has been shown

<table>
<thead>
<tr>
<th>RABBITS</th>
<th>ACTIVE STIFFNESS</th>
<th>CK-ACTIVATED STIFFNESS</th>
<th>RIGOR STIFFNESS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN±SEM (mN:mm^-2μm^-1)</td>
<td>n</td>
<td>MEAN±SEM (mN:mm^-2μm^-1)</td>
</tr>
<tr>
<td>Adult</td>
<td>755±123</td>
<td>8</td>
<td>792±122</td>
</tr>
<tr>
<td>Newborn</td>
<td>663±130</td>
<td>4</td>
<td>697±122</td>
</tr>
<tr>
<td>17 days</td>
<td>510±89</td>
<td>7</td>
<td>551±101</td>
</tr>
<tr>
<td>8 days</td>
<td>544±88</td>
<td>6</td>
<td>657±96†</td>
</tr>
<tr>
<td>3 days</td>
<td>371±68</td>
<td>3</td>
<td>595±122‡</td>
</tr>
<tr>
<td>1 day</td>
<td>200±33</td>
<td>7</td>
<td>325±62§</td>
</tr>
</tbody>
</table>

CK, creatine kinase; n, number of experiments.

*p≤0.001 compared with CK-activated stiffness by paired t test; †p≤0.05, ‡p≤0.01, and §p≤0.01 compared with active stiffness by paired t test.

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**FIGURE 4.** Graph showing sensitivity of rigor tension to substrates during rabbit left ventricular development. Normalized pMgATP/tension relations in adult rabbits (□ and ■) and in rabbits 1 (●), 3 (▲), 8 (▼), and 17 (●) days old. Rigor tension was induced by decreasing MgATP concentration in the absence (adult, open symbols) and in the presence (closed symbols) of 12 mM phosphocreatine (PCr) at pCa 9. The pMgATP/tension relation without PCr was similar in all age groups. Data were fitted using the Hill equation.
TABLE 4. Myofibrillar Creatine Kinase Efficacy as a Function of Age

<table>
<thead>
<tr>
<th>Age</th>
<th>pMgATP$_{50}$ (0 mM PCr)</th>
<th>pMgATP$_{50}$ (12 mM PCr)</th>
<th>CK efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult (n=7)</td>
<td>3.58±0.07</td>
<td>5.51±0.03</td>
<td>1.92±0.06</td>
</tr>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 days (n=5)</td>
<td>3.55±0.03</td>
<td>5.40±0.06</td>
<td>1.85±0.06</td>
</tr>
<tr>
<td>8 days (n=5)</td>
<td>3.73±0.10</td>
<td>5.18±0.08*</td>
<td>1.45±0.04*</td>
</tr>
<tr>
<td>3 days (n=5)</td>
<td>3.58±0.04</td>
<td>4.68±0.16*</td>
<td>1.10±0.14*</td>
</tr>
<tr>
<td>1 day (n=4)</td>
<td>3.78±0.06</td>
<td>4.04±0.12*</td>
<td>0.26±0.08*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. pMgATP$_{50}$, pMgATP necessary to induce half relaxation of rigor tension; PCr, phosphocreatine; CK efficacy, creatine kinase efficacy (value at 12 mM PCr minus value at 0 mM PCr).

*p≤0.001 compared with corresponding adult value.

Recently it has been found that there is a specific gene of Mt-CK for sarcomeric tissues with tissue-specific conservation among species; this indicates tissue-specific constraints that may reflect either the unique environment of mitochondria in different tissues or functional differences among isoenzymes. Thus, in rabbit heart during the first 2 weeks of life, the ability of CK to stimulate respiration progressively appears at a time of increased mitochondrial volume and oxidative metabolism.

Myocardial contractility is well known to increase during perinatal development. In skinned fibers also, both maximal force and active stiffness (Table 3) per muscle cross-sectional area increase consistently after birth with the increase in myofibrillar relative volume. These quantitative changes are accompanied by a progressive organization of contractile proteins into characteristic cross-striated myofibrils. MM-CK, the major constituent of CK in adult heart, is present as a protein associated with the M band in cardiac and skeletal muscle but may be also associated with the A band or with the I band. At birth, in the rat heart, MM-CK is found diffuse in the cytosol, and no evidence of M band nor of CK bound to the myofibrils is observed. Despite the fact that MM-CK is present in high amounts, the present results show that CK-bound enzymatic activity and functional coupling to myofibrillar ATPase are absent at birth and develop in the first 2 postnatal weeks in rabbit heart.

![Graph showing absolute parameters of mitochondrial respiration rate during rabbit left ventricular development](image1)

**Figure 6.** Graphs showing absolute parameters of mitochondrial respiration rate during rabbit left ventricular development. Panel A: Oxygen consumption rates in the absence of ADP (Vo) and in the presence of both 20 mM creatine and 1 mM ADP (Vmax) showing no significant change with age. Panel B: Oxygen consumption rates in the presence of 0.1 mM ADP (−creatine) and after subsequent addition of 20 mM creatine (+creatine). *p≤0.001 compared with adult.

![Oxygraph tracings of mitochondrial respiration during rabbit left ventricular development](image2)

**Figure 5.** Oxygraph tracings of mitochondrial respiration during rabbit left ventricular development. Oxygen consumption was determined in an oxygraph in the presence of (mM) glutamate 5, malate 2, phosphate 3, EGTA 10 (pCa 7), free Mg$^{2+}$ 3, taurine 20, dithiothreitol 0.5, imidazole 20 (pH 7.0), and 2 mg/ml bovine serum albumin, ionic strength 0.16 M with potassium 2-(N-morpholino)ethanesulfate. The arrows indicate time of addition of fibers, ADP, creatine, and carboxyatractyloside (Cat) into the medium. Fiber dry weight was 2.4 mg for adult and 2.59 mg for 1-day-old newborn.
TABLE 5. Mitochondrial Creatine Kinase Efficacy as a Function of Age

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>$V_{max}/V_o$</th>
<th>$V_C$ (%)</th>
<th>$V_{Cat}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult ($n=14$)</td>
<td>6.86±0.33</td>
<td>65.6±2.6</td>
<td>88.9±0.7</td>
</tr>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 days ($n=13$)</td>
<td>7.9±1.2</td>
<td>58.3±3.4</td>
<td>88.0±1.0</td>
</tr>
<tr>
<td>8 days ($n=14$)</td>
<td>6.01±0.44</td>
<td>42.1±3.5*</td>
<td>89.8±1.0</td>
</tr>
<tr>
<td>3 days ($n=18$)</td>
<td>5.68±0.29†</td>
<td>9.8±1.7*</td>
<td>89.5±1.2</td>
</tr>
<tr>
<td>1 day ($n=14$)</td>
<td>5.89±0.47</td>
<td>3.86±0.67*</td>
<td>85.7±1.9</td>
</tr>
<tr>
<td>Fetal ($n=4$)</td>
<td>8.00±0.57</td>
<td>3.02±0.32*</td>
<td>92.7±0.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM. $V_o$, respiration rate; $V_{max}/V_o$, acceptor control ratio (ratio of the maximal ADP-stimulated respiration rate [1 mM ADP, 20 mM creatine] to basal respiration rate); $V_C$, respiration rate increase after addition of 20 mM creatine in the presence of 0.1 mM ADP: $\%V_C=([V_C-V_{ADP}]/V_{ADP})\times100$. $V_{Cat}$, inhibition of the respiration rate by 35 μM carboxyatractylside (an index of the integrity of the inner mitochondrial membrane): $\%V_{Cat}=([V_{max}-V_{Cat}]/V_{max})\times100$.

* $p<0.001$ compared with corresponding adult value.
† $p<0.05$ compared with adult value.

At the present we do not know which factors are involved in CK binding to intracellular structures. The appearance of myofibrillar CK is associated with the maturation of myofibrils; one could suggest its relation to the appearance of the M line as shown in the rat heart after birth. In fetal hearts, both ventricles of equal mass have similar cardiac output and perform similar work. The major changes in circulation at birth (closure of arterial and venous shunts, changes in peripheral and pulmonary resistance, and modification of ventricular output) are associated with differential maturation of the two ventricles. The right ventricle undergoes a steady-state increase in its myofibrillar relative volume that persists during the first postnatal weeks, whereas an abrupt increase in myofibrillar relative volume and mass is observed in the first 4 days in the left ventricle. In the adult rat, total CK activity and distribution of CK isoenzymes differ in the left and right ventricles both in control and hypertrophied hearts. Developmental changes in CK isoenzyme distribution between the left and right ventricles are not known. Despite the opposite variations in load applied to the two ventricles after birth, the time course and the amount of CK binding to myofibrils appeared similar. Likewise, the functional activity of myofibrillar CK does not change in experimental pressure overload induced by aortic banding in the adult. Thus, at birth, the changes in blood pressure and load do not appear to be the only determinants of CK myofibrillar binding; circulating or hormonal factors could be involved. One cannot exclude that a different MM-CK isoprotein differing in its binding properties to myofibrils would be expressed after birth in parallel with the expression of Mt-CK. At the present time, there seems to be only one MM-CK gene that can be hormonally regulated. However, Boheler and Dillmann have shown that in rat heart two isoforms of MM-CK were translated in vitro and that only one of these isoforms decreased with cardiac hypertrophy, suggesting that the transcriptional or translational control of CK is much more complex than previously believed.

It is known that the activity pattern of muscle determines the fiber type and the metabolic profile. Slow and sustained activity is associated with oxidative metabolism, whereas rapid and short activity is associated with glycolytic activity. The organization of the CK system appears different in these two types of muscle; there is an abundance of cytosolic enzymes in glycolytic muscles and compartmentation of isoenzymes in oxidative muscle. The importance of the compartmentation role of CK in cardiac tissue compared with the buffering effect is illustrated by the sustained level of mechanical function observed in creatine phosphate–depleted hearts, with the small amount of PCr present being able to maintain high CK fluxes and rapid turnover of ATP and ADP. Fetal heart, because of its low $P_o_2$ environment, depends on high glycolytic and glycogenolytic activity for its ATP turnover, i.e., the high activity of cytosolic enzymes. It is worth noting that at this stage of development CK is also predominantly cytosolic, since no binding to mitochondria or myofibrils was observed. Recently, reappearance of fetal cytosolic BB-CK isozyme was found in hypertrophied adult heart together with increased glycolytic capacity. Fetal cytosolic CK could play a role of temporal and spatial buffering of adenine nucleotides similar to that described by Meyer et al in white skeletal muscle. At birth, as metabolic requirements increase,
oxidative metabolism becomes predominant in the cardiac cell. In agreement with Perry et al., total CK activity relative to protein content is constant during perinatal rabbit heart development (Figure 1A). Nevertheless, qualitative changes occur in the CK system: a mitochondrial isoform is expressed, and binding of specific isoforms takes place at the sites of energy synthesis and energy utilization. Perry et al., using 31P-magnetization transfer, showed that during the same period of development the CK reaction velocity increased as Mt-CK increased. Compartmentation of the CK system is a characteristic of aerobic muscles. This compartmentation allows rapid integration of metabolism in mitochondria and quick signaling from myofibrils to mitochondria. It also permits rapid adaptation of energy production to energy utilization, a highly necessary process in cardiac tissue. The adaptation of the newborn heart to its new environment appears very similar to the adaptation observed in chronically stimulated fast twitch rabbit muscle and in skeletal muscle of marathon runners, in which the shift from glycolytic to oxidative metabolism is accompanied by an increase in Mt-CK isoenzyme. Conversely, in rat myocardium, increased glycolytic capacity has been recently shown to be associated with increased cytosolic CK isoforms in hypertensive hypertrophy. Developing myocardium could be regarded as one example of the CK system specificity of organization depending on muscle metabolism.

The differentiation and maturation of specialized cells leads to a hierarchy of spatial organization that is structured, integrated, and compartmentalized; the appearance of integrated multi-enzyme systems constitutes a high degree of compartmentalization. This compartmentation of reactions allows an increased efficacy, since the reactions are not limited by random diffusion of products and substrates and the intermediate reactants of a metabolic chain are directed toward the active sites. Association of CK with myosin ATPase or adenine nucleotide translocase represents such two-step enzyme systems. The coimmobilization of two enzymes provides more efficient reaction than soluble enzymes: it results in shorter lag time and shorter transient times of reaction. These properties could be essential for a rapid neonatal adaptation of the myocardium to increased metabolic demand. Compartmentation of CK isoenzymes occurs during the phase of hyperplastic growth (i.e., before 8 postnatal days in the rabbit). At that time, structures involved in excitation–contraction coupling increase their complexity: enhanced calcium release and calcium ATPase activity of the sarcoplasmic reticulum provide an efficient system of calcium handling. Similarly, CK binding to intracellular structures increased the efficiency of systems involved in ATP turnover. These events parallel the decrease in the heart weight/body weight ratio in the first two postnatal weeks and could contribute to an increase in myocardial efficiency.

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