Muscle Creatine Kinase-deficient Mice

I. ALTERATIONS IN MYOFIBRILLAR FUNCTION

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The regulation of contractile activity in mice bearing a null mutation of the M-isof orm of creatine kinase gene, has been investigated in tissue extracts and Triton X-100-treated preparations of ventricular, soleus, and gastrocnemius muscles of control and transgenic mice. Skinned fiber experiments did not evidence any statistical difference in the maximal force or the calcium sensitivity of either muscle type. Rigor tension development at a low MgATP concentration was greatly influenced by phosphocreatine in control but not in transgenic mice as should be expected. In calcium-activated ventricular preparations, although the force developed by each cross-bridge was the same in control and transgenic animals, the rate constant of tension changes appeared to be markedly slowed in transgenic animals. As the ventricular isomyosin pattern was not altered, we suggested that, in transgenic animals, cross-bridge cycling was hindered by a local decrease in the MgATP to MgADP ratio, due to lack of a local MgATP regenerating system. Myokinase activity was not significantly changed while activities of pyruvate kinase or glyceraldehyde-3-phosphate dehydrogenase were found to be increased in transgenic animals. These results show that no fundamental remodelling occurs in myofibrils of transgenic animals but that important adaptations modify the biochemical pathways including glycolytic metabolism.

Creatine kinase (CK) is an important enzyme catalyzing the reversible transfer of a phosphate moiety between ATP and creatine. A major part of muscle creatine kinase exists as dimers composed of two subunits, M and B, giving three isoenzymes, MM, BB, and MB. In addition, there is a fourth isoenzyme in the mitochondria (mitochondrial CK), which differs biochemically and immunochemically from the cytosolic forms and can present octameric and dimeric structures (Wyss et al., 1992). Studies with subcellular fractionation or histochemical localization have revealed that CK isoenzymes are present in cytosol or bound to intracellular structures. M-CK has been found in myofibrils and described as a structural protein of the M-band participating in the connections between myosin filaments inside muscle fibers (Wallimann et al., 1977). Additional binding sites have been described on actin filament (Wegmann et al., 1992) or on the entire myosin filament (Otsu et al., 1989). M-CK activity in myofibrils is as high as about 2 IU/mg of protein in skeletal and ventricular muscles and represents 5% of total CK activity in fast-twitch muscle compared to 23% in ventricular muscle (Wallimann et al., 1977; Ventura-Clapier et al., 1987b; for review see Ventura-Clapier et al. (1994)). M-CK has been shown to be functionally coupled to myosin ATPase. That means that myosin ATPase preferentially uses ATP supplied by creatine kinase rather than cytosolic ATP (Bessman et al., 1980; Saks et al., 1984). Myofibrillar CK can repolyphosphorylate all of the ADP produced by myosin ATPase (Saks et al., 1976; Wallimann et al., 1984; Arrigo-Dupont et al., 1992) and can provide enough energy for maximal force and normal kinetics even in the absence of MgATP, at the expense of phosphocreatine (PCr) (Ventura-Clapier et al., 1987a; for review see Ventura-Clapier et al. (1994)).

The creatine kinase/phosphocreatine system is considered to fulfill important roles in the energy metabolism of skeletal and cardiac muscles (for reviews see Wallimann et al. (1992), Wyss et al. (1992), and Saks et al. (1994)). In skeletal muscles, activity pattern determines fiber type and metabolic profile. Fast-twitch (white) muscles exhibiting rapid and brief activity patterns are mainly glycolytic and contain high amounts of PCr and CK (Iyengar, 1984; Yamashita and Yoshioka, 1991). By contrast, slow-twitch (red) skeletal muscle or cardiac muscle, exhibits prolonged and sustained activity associated with well developed oxidative metabolism and relatively low contents of PCr and CK. The organization of the CK system appears different in these two kinds of muscles; there is an abundance in cytosol of the muscle form of creatine kinase (M-CK) enzyme in fast-twitch muscle and compartmentation of the mitochondrial and M-isoenzymes in slow-twitch muscle and ventricle.

Functional consequences and adaptive strategies observed in animal models of long term deficiency in the CK system may give insights into the physiological role of this system in different muscle types. Long term alterations in the creatine kinase/phosphocreatine system have been developed by feeding animals with slowly metabolized analogues of creatine (β-guanidinopropionic acid, β-GPA). These animals exhibit decreased PCr and ATP concentrations in cardiac and skeletal muscles (Shoubridge and Radda, 1984; Kapelko et al., 1988; Zweier et al., 1991). In addition, a clear cardiac hypertrophy and isoenzyme shift from fast V1 to slow V3 myosin have been observed (Mekhfi et al., 1990). Adaptive strategy in the heart is directed toward an increase in the number of contractile units together with an increased efficiency of each unit to respond to de-
creased metabolic fluxes. In the same animal model, skeletal muscles exhibited an enhanced oxidative metabolism and an isomyosin shift from fast- to slow-type isomyosins (Shoubridge et al., 1985; Moerland et al., 1989).

More recently, a mouse line bearing a null mutation of M-CK has been developed (van Deursen et al., 1993). Genetic M-CK knocking out is a unique model of complete isoenzyme-specific muscle form of CK, which allows us to investigate myofibrillar properties without interference with cytosolic substrate and ion changes. The results show that intrinsic mechanical capacities and calcium sensitivity were maintained in transgenic animals, although skinned fibers were not able to utilize PCr. However, contractile kinetics were markedly slowed down despite an unchanged myosin isoenzyme profile. In addition, the energy supply pattern was changed since glycolytic capacity seemed to increase in fast-twitch muscle as well as in ventricular muscle.

Materials and Methods

Mouse Model of Muscle Creatine Kinase-deficient Mice

Mice bearing a null mutation of the M-CK gene were obtained as described previously (van Deursen et al., 1993). Heterozygous mutants were interbred to generate mice deficient in M-CK. Offspring were genotyped 2 weeks after birth.

Six control adult female mice C57BL/6 and 5 adult transgenic mice were anesthetized with an intraperitoneal injection of pentobarbitone according to the recommendations of the Institutional Animal Care Committee (INSERM, Paris, France) and weighed. While under anesthesia, animals were exsanguinated, and various organs were isolated and weighed. Heart, gastrocnemius, and soleus muscles were placed in a modified Krebs solution containing (mM): NaCl, 118; KCl, 4.7; NaHCO3, 25; KH2PO4, 1.2; and MgSO4, 1.2.

Organ samples were frozen for further analysis. Other samples were minced with scissors, placed into cold solution (50 mg wet weight per 1 mL) containing (mM): K2HPO4, 100 (pH 8.7); EGTA, 1; N-acetyl cysteine, 15; and homogenized in a Ultra-Turrax homogenizer. Tissue homogenates were incubated for 60 min at 0°C for complete extraction of CK and other enzymes, centrifuged at 13,000 x g for 20 min, and the supernatant was used for determination of CK and myokinase and frozen.

Mechanical Experiments

Muscle Preparation—Muscle fiber bundles were dissected from soleus or gastrocnemius from papillary muscles of the left ventricle in mice in a zero-Ca2+ Krebs solution, pH 7.4. Bundles were incubated for 1 h 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 and kept at 4°C until use. After the skimming procedure, one bundle was snared at both ends with hair emerging from stainless steel tubes in the experimental apparatus. It was adjusted to slack length, stretched by 20%, and subjected to a series of tension cycles. Subsequent length was controlled by laser diffraction (10-milliwatt He-Ne laser Spectro-Physics, Inc., Mountain View, CA). The length and diameter of the muscles were measured by use of a graticule in the dissecting microscope. Muscles were immersed in 2.5-mL chambers arranged around a disk in a temperature-controlled bath positioned on a magnetic stirrer. Each solution was well-stirred at high speed. All experiments were performed at 22°C.

Experimental Apparatus—The tubes were connected to a transducer (model AB 801, Sensonor, Microelectronics, Horten, Norway) and a vibrator as described previously (Mayoux et al., 1994). The bandwidth of the transducer and tube was 2 kHz. The permanent magnet and coil came from a standard loudspeaker (Pioneer TS-130A, Pioneer Electric and Research Corp., Forest Park, IL). The coil was glued to a glass tube (2 mm in diameter) driven in an axial ball bearing (total moving mass < 1.5 g). A flag with a narrow window was glued on the glass axis between a lamp and a position detector (type Si543, 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 ms without overshoot. Length and tension changes were monitored on a digital storage oscilloscope (OS4020, Gould, Inc., Cleveland, OH). Tension tracings were digitized at 20 kHz (12-bit analog/digital converter), analyzed on-line using a PC compatible computer, and stored on a videotape.

Solutions—Solutions were calculated using the computer program of Fabiato (1988). All solutions were calculated to contain (mM): EGTA, 10; imidazole, 30; Na+, 30.6; Mg2+, 3.16; and dithiothreitol, 0.3; ionic strength was adjusted to 0.18 m with potassium acetate. pH was adjusted to pH 7.1 with acetic acid. In relaxing agonist solutions, pCa was 9. In activating solution, pCa was 4.5. Relaxing and activating solutions also contained 3.16 mM MgATP and 12 mM PCr. Rigor solutions were obtained by mixing two solutions of pMgATP 2.5 and 6 or pMgATP 4 and 6. EGTA was obtained from Sigma. PCR (Neoton, Schiffarelli Farmaeutica SEARLE, Turin, Italy) was a kind gift of Prof. E. Strumia.
pH 7.6 in a medium containing (in mM): triethanolamine, 82.5; phospho
enolpyruvate, 0.54; MgSO₄, 2.5; KCl, 10; ADP, 4.7; NADH, 0.2; and 9.2 IU/ml lactate dehydrogenase. Fructose-6-phosphokinase was determined at pH 8.5 in a medium containing (in mM): Tris-HCl, 70; MgSO₄, 1.4; KCl, 4.5; phosphoenolpyruvate, 0.71; fructose-1,6-diphosphate, 0.64; fructose-6-phosphate, 1.8; ATP, 1.1; NADH, 0.2; and 8.6 IU/ml lactate dehydrogenase and 4.2 IU/ml pyruvate kinase. Lactate dehydrogenase activity was determined at pH 7.4 using a medium containing (in mM): KH₂PO₄, 20; KCl, 120; dithiothreitol, 0.5; pyruvate, 10; NADH, 0.2.

**Myosin Isoforms**

**Myosin Preparation**—Frozen muscles were thawed on ice, cut into small pieces, and washed with 5 volumes of 20 mM NaCl, 5 mM sodium phosphate, and 1 mM EGTA (pH 6.5). Myosin was then extracted with 3 volumes of 100 mM sodium pyrophosphate, 5 mM EGTA, and 1 mM dithiothreitol (pH 8.5); after 30 min of gentle shaking, the mixture was centrifuged at 10,000 × g. The supernatant containing myosin was diluted with 1 volume of glycater and stored at −20 °C (d’Albis et al., 1979).

**Electrophoresis of Native Myosin Isoforms**—Gel running buffer con­sisted of 20 mM sodium pyrophosphate (pH 6.5), 10% glycerol, 0.01% 2-mercaptoethanol, and 2 mM MgCl₂. Cylindrical (6 × 0.5 cm) gels contained 4% polyacrylamide (3.88% acrylamide and 0.12% N,N'-methylenebisacrylamide). Between 1 and 5 μg of myosin was loaded on each gel. Electrophoresis was carried out at a constant voltage of 90 V, for 22 h, between 0 and 2 °C (d’Albis and Gratzer, 1973; Hoh, 1975).

**Electrophoresis of Myosin Heavy Chains and Quantification**—Electrophoresis was performed as described by Talmadge and Roy (1993). Minigels were used in the Bio-Rad Mini-PROTEAN II Dual Slab Cell. Electrophoresis took place in a cold cupboard, at 10 °C for 28 h. The gels were stained with Coomassie Blue R-250, and the relative amounts of the different myosin heavy chains were measured using a densitometer equipped with an integrator.

**Statistical Analysis**

Values were expressed as mean ± S.E. Student’s t test was used to compare the means between control and transgenic animals or inside groups between two experimental conditions. Statistical significance was reached when p < 0.05.

**RESULTS**

**Anatomical Data**—Comparing control and transgenic mice, no statistical difference was observed between heart, lung, liver, kidney, and body weights (Table I). The heart weight to body weight ratio was in consequence not altered in transgenic animals. Thus, no sign of organ dysfunction or change in muscle mass or cardiac insufficiency could be detected.

**Tension and Calcium Sensitivity of Skinned Fibers**—Fibers of similar diameter were dissected from control and transgenic mice. Diameters were, respectively, for control and transgenic: 207 ± 23 and 179 ± 16 μm for ventricle, 160 ± 15 and 138 ± 20 μm for soleus, 156 ± 26 and 170 ± 26 μm for gastrocnemius. No statistical difference was observed, which allowed accurate comparison of mechanical performances. Table II shows mechanical parameters of skinned fibers, normalized per cross-sectional area, in resting (pCa 9) and activating (pCa 4.5) conditions. Whatever the muscle, resting tensions were not modified in transgenic animals. Although clear differences existed between active forces developed by the different muscles, no differences were observed between control and transgenic animals.

Each fiber bundle was submitted to a set of solutions of increasing calcium concentrations (see “Materials and Methods”), and pCa/tension relations were calculated according to the Hill equation. Mean pCa for half-maximal activation (pCa₅₀) and n values are reported in Table II. No significant change in calcium sensitivity could be detected except a small increase in Hill coefficient in gastrocnemius muscle.

It is known, however, that inactivation of myofibrillar CK, either by inhibition or in the absence of PCr, leads to a change in the calcium/tension relationship. This was confirmed in car­diac fibers of control mice (Fig. 1) where omission of PCr in activating solution led to an increase in calcium sensitivity from 5.68 ± 0.03 to 5.98 ± 0.03 (n = 4, p < 0.001).

**Effect of Phosphocreatine on Relaxation of Rigor Tension**—The next series of experiments was undertaken to study the influence of PCr on the relaxation of rigor tension in control and transgenic muscles. Rigor tension is the tension induced in the virtual absence of calcium by decreasing MgATP concentration. We have shown in ventricular muscle that the develop­ment of rigor tension is greatly influenced by CK bound in myofibrils. The concentration of MgATP necessary to obtain half-maximal rigor is greatly reduced when PCr is provided as a substrate. In order to characterize more precisely this effect in control and transgenic mice, complete pMgATP/rigor tension relations have been established in presence or absence of PCr for sets of solutions of decreasing MgATP concentrations. In Fig. 2, pMgATP/rigor tension relations have been drawn using Hill equation and pMgATP₅₀ (pMgATP for half-maximal rigor force) values given in Table II for ventricle, soleus, or gastro­cnemius. When PCr was added, a clear shift of the relation was observed in control muscles. While pMgATP/tension relations without PCr were identical in control and transgenic animals, no shift could be evidenced in the presence of PCr for the three muscle types of transgenic animals. pMgATP₅₀ values in the presence of PCr, as well as CK efficacy, which is defined as the difference between pMgATP₅₀ values in the presence and absence of PCr, were both highly significantly different between control and transgenic mice (Table II). This result is in complete agreement with the absence of M-CK in these animals and clearly shows that myofibrils from transgenic mice have no enzyme able to utilize PCr.

**Responses of Ventricular Skinned Fibers to Quick Length Changes**—Active stiffness as measured by the tension re­ponses to a quick length change was not significantly different in ventricular fiber bundles of control and transgenic mice (450 ± 57 mN/mm/mm² (n = 8) versus 644 ± 69 mN/mm/mm² (n = 6) respectively). It should be noted, however, that a small trend toward an increase in both tension and stiffness could be observed, although not reaching significance, possibly as a result of poor estimation of the fibers’ effective cross-section. However, the ratio of force to stiffness was not altered (Fig. 3) in transgenic mice showing that force developed by each cross-bridge was preserved. When a length perturbation was applied to activated muscle, force first increased in phase with the increase in length and then decreased toward the value of the tension
before the length change. The rate constant of tension recovery reflects the cross-bridge cycling rate, while the extent of recovery characterizes the cross-bridge state. The rate constant of tension recovery following stretches was greatly decreased in transgenic mice compared to control while the extent of recovery was not different (Fig. 3). This result suggests a decrease in the cross-bridge cycling rate in transgenic ventricular fibers.

Indeed, inhibition of myofibrillar CK slows down tension kinetics in skinned rat cardiac muscle, probably due to local accumulation of protons and MgADP (Ventura-Clapier et al., 1987b). This was also observed in mouse heart where with­drawal of PCr decreased the rate constant from 103 ± 17 s⁻¹ to 38 ± 4 s⁻¹ (n = 4, p < 0.05).

Isomyosin Patterns—A decrease in cross-bridge cycling rate may arise from an altered pattern in myosin isoenzyme distribution. In order to see if transgenic animals exhibit a change in myosin isoforms, the myosin phenotype was determined in the different muscles.

Two types of gel electrophoresis were used to analyze the content in myosin isoforms of the cardiac and skeletal muscles, respectively. The cardiac ventricular myosins are best separated by gel electrophoresis under nondissociating conditions (Lompré et al., 1981), as shown here in a control rat heart, which displayed the three isoforms V1, V2, and V3 (Fig. 4a).

Both control and transgenic mouse ventricles displayed only the V1 isoform.

To analyze the myosin isofrom content in the skeletal muscles, gel electrophoresis in the presence of SDS allowed the separation of the slow type isoform 1 and the three fast type isoforms 2 (Fig. 4b). The only transgenic mouse soleus muscle contained the 2A and the 1 isoforms in the same proportions, 35% and 65%, respectively, as the control muscle. The gastro-

**Figure 1.** Original tension recording of the responses of a control mouse skinned ventricular fiber to an increase in calcium concentration in the presence or in the absence of PCr. Letters represent different pCa values: a; b, 6.25; c, 6.125; d, 6; e, 5.875; f, 5.75; g, 5.625; h, 5.5; i, 5.375; j, 5.5. Diameter of the fiber was 210 μm. Resting tension was 4.88 mN/mm². Tₐₐₐ was 80.1 mN/mm². pCₐₐ and Hill coefficient were, respectively, 5.72 and 5.04 in the presence and 6.01 and 1.51 in the absence of PCr. Notice that active tension developed for lower calcium concentrations in the absence of PCr.
In this study, attempts were made to characterize the intrinsic properties of cardiac and skeletal myofibrils of mice bearing a null mutation for the M-form of CK. Skinned fiber technique was used to destroy cellular membranes, while keeping the cellular architecture intact, so that intrinsic mechanical properties of the myofibrillar network, in a definite medium surrounding myofibrils, could be investigated. The results showed that maximal force and stiffness characteristics were not altered while kinetics of force changes assessed in ventricular tissue were markedly reduced despite an unchanged isomyosin profile. Sensitivity to added ATP was not altered, while addition of PCr was without effect in mutants, suggesting no unknown route for PCR utilization inside myofibrils. Increased glycolytic activity could be one possible adaptational way to control the ATP/ADP ratio inside myofibrils devoid of bound CK during contraction.

Muscle contraction is the result of cyclic association between the thin and thick filaments resulting in the relative sliding of these filaments past each other when muscle is allowed to shorten, or resulting in force development in isometric conditions. This mechanical interaction or cross-bridge cycling is coupled to the hydrolysis of ATP to ADP by myosin ATPase located on the thick filament and regulated by the binding of calcium to the troponin complex of the thin filament. The products of ATP hydrolysis are released when myosin is attached to actin during the power stroke portion of the cycle, and an increase in hydrolytic products such as ADP, inorganic phosphate, and H⁺ is expected to influence the different steps and thereby the power stroke. Earlier studies have shown that MgADP increases isometric tension and calcium sensitivity and decreases maximal velocity of shortening or kinetics of force development (Brandt et al., 1982; Cooke and Pate, 1985; Ventura-Clapier et al., 1987a; Hoar et al., 1987). ADP detachment is considered to be the rate-limiting step in cross-bridge detachment and for the overall cross-bridge cycle (Siemankowski et al., 1986). ADP accumulation may inhibit the interaction between actin and myosin by competing with MgATP at the active site of the myosin molecule, thus slowing down MgADP detachment and further MgATP binding and cross-bridge detachment (for review see Ventura-Clapier et al. (1994)).

The rate constant of tension recovery following stretches is an estimate of the kinetics of cross-bridge cycling and reflects the rate-limiting step in the cycle; it was shown to vary with myosin isomorph composition as well as following alterations in concentrations of substrates or products of myosin ATPase (Ventura-Clapier et al., 1987a; Mekhfi and Ventura-Clapier, 1988; Mayoux et al., 1994). We have observed a 3-fold decrease in cross-bridge cycling rate in cardiac myofibrils of transgenic animals compared to control without any shift in myosin isomers. A similar change was observed in control mice when PCr was omitted in the solution. Thus, this decreased rate of force changes can be attributed to changes in ATP/ADP ratio in the vicinity of myosin ATPase with a consequent product inhibition of ATPase activity. Accumulation of MgADP as a result of a lack of myofibrillar CK will induce an increase in force production and a decrease in rate of cross-bridge cycling, leading to a lower energy consumption and better economy of force production. As a consequence, the rates of force production and relaxation of the muscle twitch would be decreased. However, for cardiac muscle having cyclic activity, this would tend to increase the end-diastolic pressure and to decrease the ventricular filling, except if the intrinsic heart rate is decreased. Unfortunately, no information are as yet available concerning heart rate, developed pressure, or the force-length relationship.
of cardiac muscle in transgenic animals. Although the cross-
bridge cycling rate of skeletal muscle could not be determined in
this study, it is highly probable that tension kinetics would be
slowed also. Further studies are needed to clarify the con-
traction kinetics of the intact muscles in these animals.

In Triton X-100-treated fibers, loosely bound enzymes are
usually detached from the myofibrillar structures. In intact
cells, many enzymes including glycolytic enzymes, AMP deami-
nases, and myokinase are bound to myofibrillar proteins, mainly
to the thin filament, and may participate in MgADP/MgATP
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as has been shown under the influence of hypothyroidic treatment (Ng et al., 1991). The main difference between the two models is that ATP as well as PCr contents are preserved in the case of the M-CK mutation in comparison with β-GPA feeding where both compounds appear to be decreased. A consequence of this would be that the expression of proteins of the contractile apparatus is more under the control of the concentrations of metabolites. On the other hand, it should be borne in mind that targeted mutations affect the animals in the early embryonic life where the potentialities for adaptations are much larger than in the adult animals and may have involved more integrated adaptation mechanisms.

NMR experiments showed that fluxes through CK were not detectable in skeletal muscle of CK-deficient mice until a threshold of activity was reached (van Deursen et al., 1993, 1994). This suggested that bound CK fluxes are NMR invisible and that thorough examination considering the dynamic of life, more may be learned from the ever, since functions considered as essential for life could be detectable in skeletal muscle of CK-deficient mice until a threshold of activity was reached (van Deursen et al., 1993, 1994). This suggested that bound CK fluxes are NMR invisible and that thorough examination of biochemical and physiological characteristics of these animals would be of potentially high significance in the understanding of the role of a given pathway.

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REFERENCES