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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-isoform 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 bioenergetic pathways including glycolytic metabolism.

Creatine kinase (CK)[1] 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 rephosphorylate all of the ADP produced by myosin ATPase (Saks *et al.*, 1976; Wallimann *et al.*, 1984; Arrio-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 V_1 to slow V_3 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-

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[1] The abbreviations used are: CK, creatine kinase; M-CK, muscle form of creatine kinase; β-GPA, β-guanidinopropionic acid; PCr, phosphocreatine.

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 CK deficiency in contrast to GPA feeding, a model of substrate deficiency. Another important difference between these two models is that mutant muscles keep mitochondrial and, in principle, brain isoforms of CK which could participate in energy metabolic pathways. It was shown that M-CK deficiency does not lead to compensatory overexpression of other CK isoenzymes. Muscles from these mice, which do not express the muscle form of CK, are able to use PCr but lack the ability to perform burst activity. In order to get insights into the functional characteristics and possible adaptational processes at the level of myofibrils in these transgenic mice, we characterized intrinsic mechanical properties of ventricular, soleus, and gastrocnemius muscles using the skinned fiber technique 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. Offsprings 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 anesthetics, 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; $NaHCO_3$, 25; KH_2PO_4, 1.2; and $MgSO_4$, 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): K_2HPO_4, 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 × *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 or from papillary muscles of the left ventricle of mice in a zero-Ca^{2+} Krebs solution, pH 7.4. Bundles were incubated for 1 h in a relaxing solution (*p*Ca 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 skinning 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 an activation/relaxation cycle. Sarcomere length was controlled by laser diffraction (10-milliwatt He-Ne laser, Spectra-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 AE 801, SensoNor Microelectroniks, 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 axis (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 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 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; Mg^{2+}, 3.16; and dithiothreitol, 0.3; ionic strength was adjusted to 0.16 M with potassium acetate. pH was adjusted to pH 7.1 with acetic acid. In relaxing and rigor solutions, *p*Ca was 9. In activating solution, *p*Ca 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 *p*MgATP 2.5 and 6 or *p*MgATP 4 and 6. EGTA was obtained from Sigma. PCr (Neoton, Schiapparelli Farmaceutica SEARLE, Turin, Italy) was a kind gift of Prof. E. Strumia.

Experimental Protocols

pCa/Tension Relations—*p*Ca/tension relations were determined under isometric conditions by briefly placing each fiber into solutions of increasing calcium concentration until maximal tension was reached. Data were fitted using linearization of the Hill equation for relative tensions above 10% and under 90% as follows: $T = [Ca^{2+}]^n/(K + [Ca^{2+}]^n)$ were T is relative tension, K a constant, and n the Hill coefficient. n and *p*Ca for half-maximal activation ($pCa_{50} = -\log_{10}K/n$) were calculated for each fiber by means of linear regression analysis. Resting tension was measured at *p*Ca 9. Active tension (expressed as mN/mm^2) was the total tension at *p*Ca 4.5 minus resting tension.

pMgATP/Rigor Tension Relations—*p*MgATP/rigor tension relations were established by stepwise decreasing ATP concentrations until maximal rigor tension was obtained. The fiber was then placed in the relaxing solution for 10 to 15 min before a new set of rigor solutions was applied. Data were fitted using the Hill equation. The *p*MgATP for half-maximal rigor tension, $pMgATP_{50} = (-\log_{10}K)/n$, was calculated for each experimental condition using linear regression analysis.

Stiffness and Kinetic Measurements—To determine fiber bundle stiffness and the rate constant of tension recovery in cardiac preparations, quick length changes (0.3–3% of initial muscle length) were applied in the relaxing and activating solutions. Twelve successive stretches and releases were made, starting with the relaxing solution. Only responses to stretches were used for calculations. Each value for each fiber was the mean of five to seven stretches of varying amplitudes performed in a given experimental condition. The spike of tension in phase with the length change characterized the elastic phase (Huxley and Simmons, 1971). Stiffness was the extreme tension reached during stretching (mN/mm^2) divided by the length change (μm). A first series of length changes was imposed in the relaxing solution to assess passive properties of each fiber. Resting stiffness was calculated by linear regression analysis on the responses to stretches. Then a second series of length changes was initiated in control activating solution. The tension level before the first stretch was taken as the maximal tension and used for normalizations. Active or rigor stiffness were calculated as the difference between total stiffness minus resting stiffness. The rate constant of tension recovery after quick stretches was calculated by a least square regression analysis, according to a single exponential model, between 50% and 80% of recovery and using stretches of more than 1%.

Enzyme Analysis

Enzyme activities were determined spectrophotometrically at 340 nm (Gilford Spectrophotometer, Corning, NY), 30 °C, by using coupled enzyme systems. Results are given in IU/g wet weight. Myokinase activity was determined at pH 7.1 using the coupled enzyme assay of hexokinase and glucose-6-phosphate dehydrogenase producing NADPH. Activity was assayed in a medium containing (in mM): HEPES, 20; $MgCl_2$, 5; dithiothreitol, 0.5; ADP, 1.2; glucose, 20; NADP, 0.6; and 2 IU/ml glucose-6-phosphate dehydrogenase and hexokinase. Glyceraldehyde-3-phosphate dehydrogenase was determined at pH 7.6, in a medium containing (in mM): triethanolamine, 82.5; 3-phosphoglycerate, 6; ATP, 1.1; EDTA, 0.9; $MgSO_4$, 1.7; NADH, 0.2; and 15 IU/ml 3-phosphoglycerate kinase. Pyruvate kinase activity was measured at

TABLE I
Anatomical data of control and M-CK-deficient mice

Values are mean ± S.E.

	Body	Heart	Liver	Kidney	Lung	HW/BW[a]
	g	*mg*	*mg*	*mg*	*mg*	*mg/g*
Control (5)[b]	27.0 ± 1.3	155 ± 14	1223 ± 94	376 ± 28	171 ± 14	5.73 ± 0.34
Transgenic (5)	25.3 ± 1.0 (NS)[c]	135 ± 6 (NS)	1145 ± 88 (NS)	347 ± 19 (NS)	178 ± 9 (NS)	5.34 ± 0.11 (NS)

[a] HW/BW, heart weight/body weight ratio.
[b] Numbers in parentheses, number of animals.
[c] NS, not significant.

pH 7.6 in a medium containing (in mM): triethanolamine, 82.5; phosphoenolpyruvate, 0.54; $MgSO_4$, 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; $MgSO_4$, 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 9.6 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): KH_2PO_4, 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 glycerol 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 8.5), 10% glycerol, 0.01% 2-mercaptoethanol, and 2 mM $MgCl_2$. 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, 155 ± 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 (*p*Ca 9) and activating (*p*Ca 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 *p*Ca/tension relations were calculated according to the Hill equation. Mean *p*Ca for half-maximal activation (pCa_{50}) 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 *p*MgATP/rigor tension relations have been established in presence or absence of PCr in sets of solutions of decreasing MgATP concentrations. In Fig. 2, *p*MgATP/rigor tension relations have been drawn using Hill equation and $pMgATP_{50}$ (*p*MgATP 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 *p*MgATP/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. $pMgATP_{50}$ values in the presence of PCr, as well as CK efficacy, which is defined as the difference between $pMgATP_{50}$ 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 responses to a quick length change was not significantly different in ventricular fiber bundles of control and transgenic mice (450 ± 57 mN/μm/mm^2 ($n = 8$) *versus* 644 ± 69 mN/μm/mm^2 ($n = 6$) respectively). It should be noted, however, that a small tendency 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

TABLE II

Mechanical characteristics of skeletal and cardiac skinned fibers from control and M-CK-deficient mice

Values are mean ± S.E.

	Resting tension	Active tension	pCa_{50}	n_H	$pATP_{50}$	$pATP_{50}$ + PCr	CK_{eff}[a]
	mN/mm^2	mN/mm^2					
Ventricle							
Control	3.35 ± 0.68 (8)[b]	37.9 ± 4.4 (8)	5.723 ± 0.041 (10)	2.83 ± 0.16 (10)	3.44 ± 0.07 (8)	4.99 ± 0.05 (8)	1.55 ± 0.09 (8)
Transgenic	3.27 ± 0.60 (7) (NS)[c]	47.2 ± 3.3 (7) (NS)	5.761 ± 0.027 (7) (NS)	2.75 ± 0.22 (7) (NS)	3.47 ± 0.08 (8) (NS)	3.58 ± 0.03[d] (8)	0.11 ± 0.06[d] (8)
Soleus							
Control	5.82 ± 0.63 (6)	103 ± 21 (6)	5.825 ± 0.074 (8)	3.12 ± 0.28 (8)	3.65 ± 0.07 (8)	4.90 ± 0.03 (8)	1.25 ± 0.07 (8)
Transgenic	6.99 ± 1.81 (6) (NS)	123 ± 23 (6) (NS)	5.745 ± 0.052 (6) (NS)	4.00 ± 0.68 (6) (NS)	3.87 ± 0.07 (5) (NS)	3.92 ± 0.10[d] (5)	0.05 ± 0.06[d] (5)
Gastrocnemius							
Control	13.3 ± 6.1 (4)	275 ± 47 (6)	5.849 ± 0.082 (8)	3.20 ± 0.29 (8)	3.43 ± 0.08 (6)	4.98 ± 0.10 (6)	1.56 ± 0.06 (6)
Transgenic	7.8 ± 2.0 (7) (NS)	155 ± 28 (7) (NS)	5.937 ± 0.043 (7) (NS)	5.10 ± 0.59[e] (7)	3.55 ± 0.05 (8) (NS)	3.57 ± 0.10[d] (8)	0.02 ± 0.09[d] (8)

[a] CK_{eff}, efficacy of creatine kinase expressed as a $pMgATP_{50}$ value at 12 mM PCr minus the value at 0 mM PCr.
[b] Numbers in parentheses, number of fibers.
[c] NS, not significant.
[d] $p < 0.001$ relative to respective value for fibers of control mice.
[e] $p < 0.05$ relative to respective value for fibers of control mice.

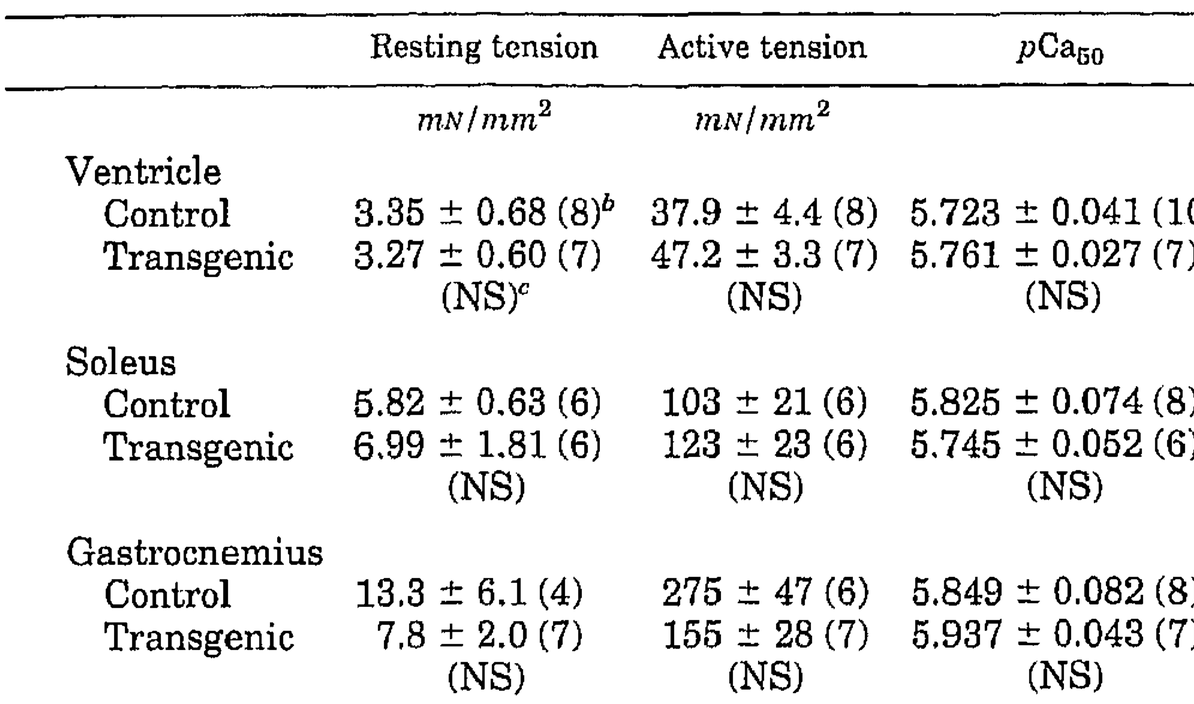

FIG. 1. **Original tension recording of the responses of a control mouse skinned ventricular fiber to an increase in calcium concentration in the presence or in the absence of PCr.** *Letters* represent different pCa values: *a*, 9; *b*, 6.25; *c*, 6.125; *d*, 6; *e*, 5.875; *f*, 5.75; *g*, 5.625; *h*, 5.5; *i*, 5.375; *j*, 4.5. Diameter of the fiber was 210 μm. Resting tension was 4.88 mN/mm². T_{max} was 30.1 mN/mm². pCa_{50} and Hill coefficient were, respectively, 5.72 and 2.04 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.

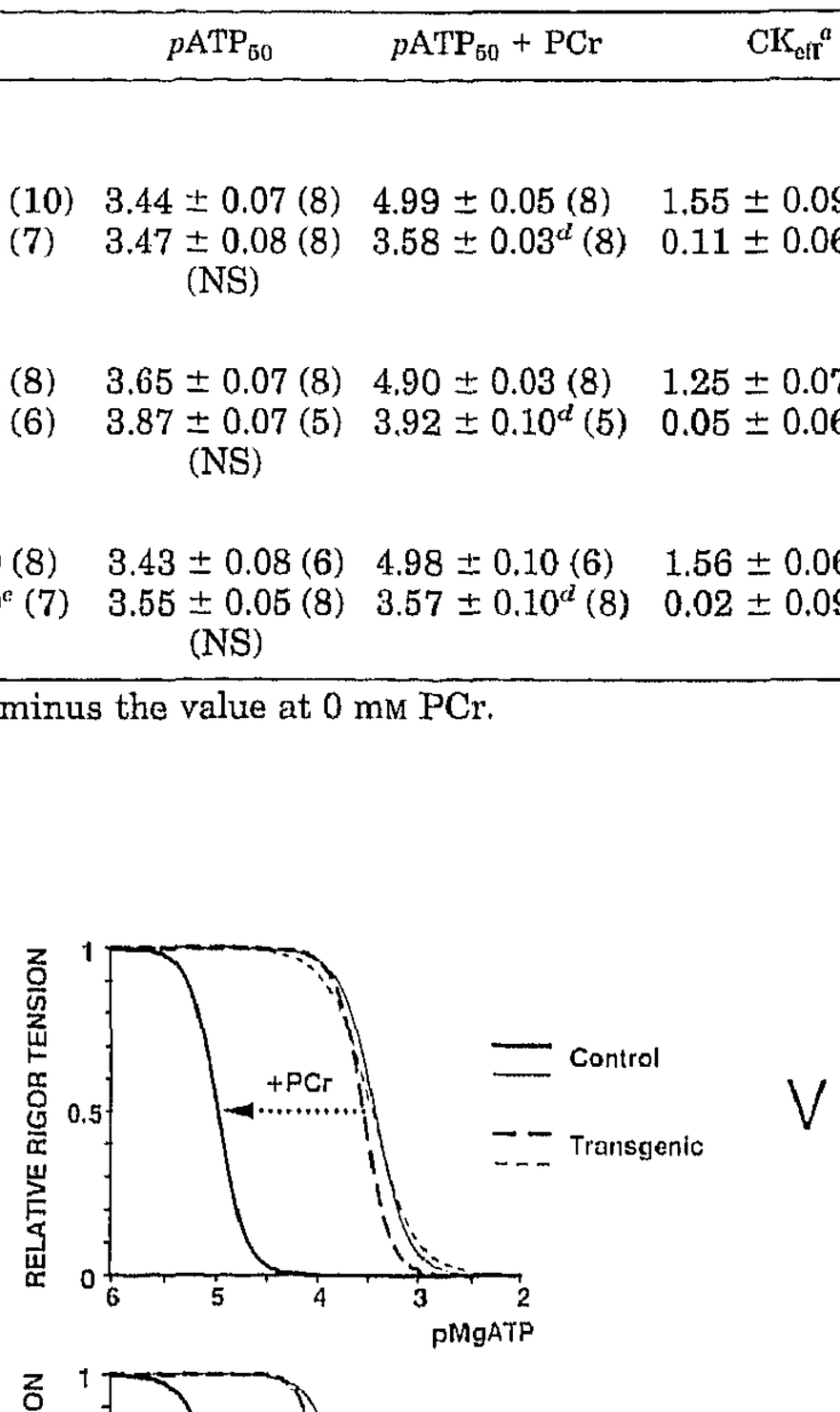

FIG. 2. **Graphs showing relative pMgATP/rigor tension relations with or without PCr, obtained in ventricular (*V*), soleus (*S*), and gastrocnemius (*G*) skinned fibers from control (*continuous lines*) and mutant mice (*dashed lines*).** $pMgATP$ *versus* rigor tension relations were calculated and plotted according to the Hill equation $T = K/(K + [MgATP]^n)$, where T is relative tension, K is a constant, and n the Hill coefficient. Each curve was drawn using the means of the n values and $pMgATP$ for half-maximal tension ($pMgATP_{50}$) calculated for each fiber and averaged in Table II. *Arrows* indicate CK efficacy values in control animals. Addition of PCr (*solid lines*) was able to shift the $pMgATP$/tension relation of all muscles of control animals but not of mutant animals.

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. 4*a*). Both control and transgenic mouse ventricles displayed only the V1 isoform.

To analyze the myosin isoform 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. 4*b*). 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-

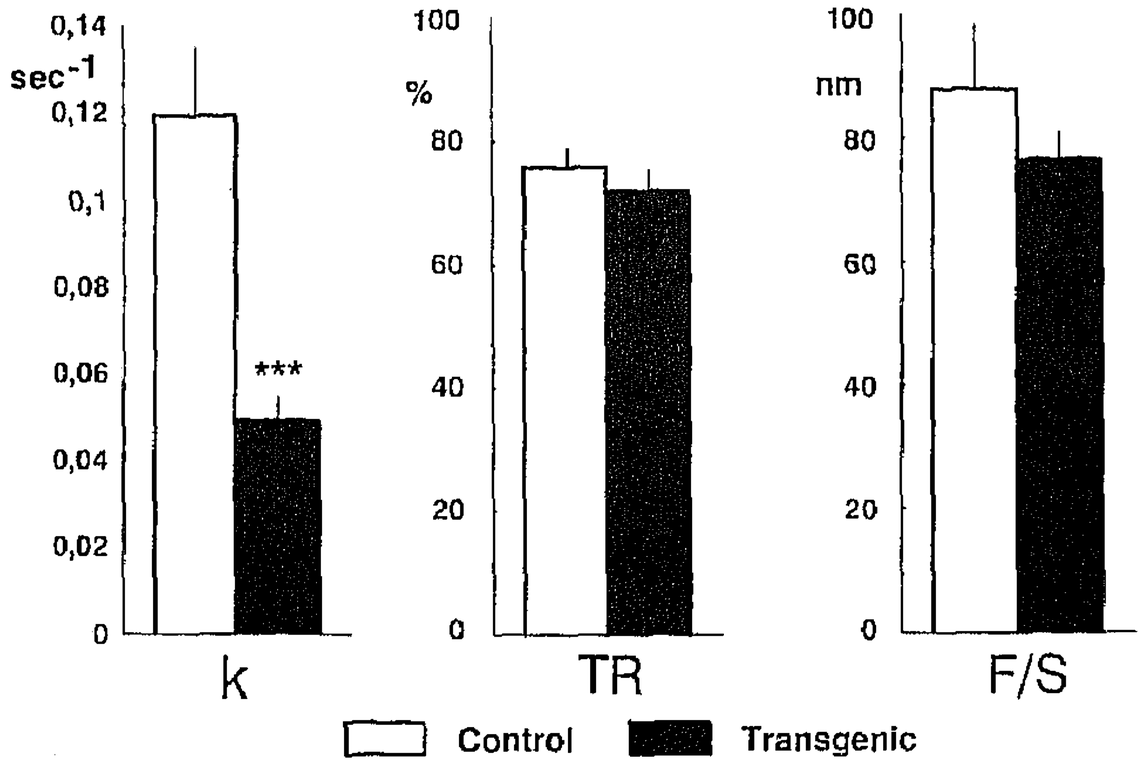

FIG. 3. **Mechanical characteristics of ventricular skinned fibers from control and transgenic mice measured using the quick length change technique.** *k* is the mean of the rate constants of tension changes following stretches of different amplitudes; *TR* is the level of tension recovery following stretches expressed in percent of tension changes; *F/S* is the force to stiffness ratio determined for each fiber. Only the rate constant of tension changes was decreased in mutant mice while the other properties were preserved.

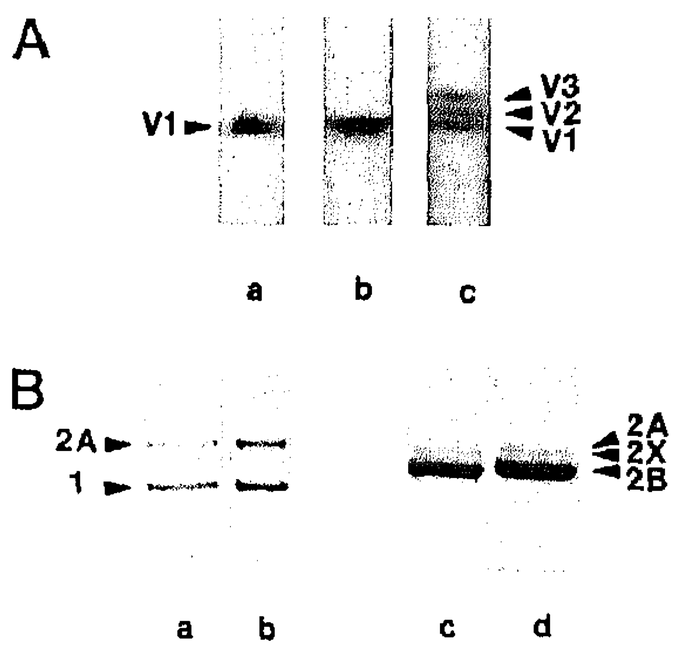

FIG. 4. *A*, gel electrophoresis of cardiac ventricular native myosin. *a*, control mouse ventricle. *b*, transgenic mouse ventricle. *c*, rat ventricle. *V1*, *V2*, and *V3* designate the three types of ventricular myosin. *B*, myosin heavy chains of soleus and gastrocnemius muscles. *a*, control mouse soleus. *b*, transgenic mouse soleus. c, control mouse gastrocnemius. *d*, transgenic mouse gastrocnemius. *2A*, *2X*, *2B*, and *1* designate the four types of skeletal muscle myosin heavy chains.

cnemius muscles mainly contained the 2B isoform, 94 ± 2% (n = 4) in the control mice and 89 ± 2% (n = 4) in the transgenic mice; the difference was not significant. The remaining myosin was distributed between a trace of the slow-type 1 isoform and the two other fast-type 2A and 2X isoforms.

Glycolytic Enzymes and Myokinase Determinations—Since no change in isomyosin pattern could be detected in muscles of transgenic mice, the question arose as to how MgATP and MgADP concentrations could be controlled in transgenic animals. Indeed, it has been shown that myofibrillar CK, by keeping high ATP/ADP ratio and low proton concentrations close to myosin ATPase, ensures optimal efficiency of myosin ATPase in skeletal (Bessman *et al.*, 1980) as well as cardiac muscle (Ventura-Clapier *et al.*, 1987a, 1987b). Other MgATP regenerating enzymes may exist in cytosol or may be loosely bound to myofibrils. To check for possible overexpression of such enzymes, glycolytic enzymes as well as myokinase activities were measured in the different muscles. Results are shown in Table III. Myokinase activity was not significantly increased in transgenic animals while some glycolytic enzymes like pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase increased in ventricles and glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase increased in gastrocnemius. No significant changes were seen for soleus muscle.

DISCUSSION

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.*, 1985). 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 isoform 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 isoforms. 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

TABLE III

Energy metabolism of skeletal and cardiac muscle from control and M-CK-deficient mice

Values are expressed in IU/min/g wet weight. Values are mean ± S.E.

	Myokinase	Fructose-6-phosphokinase	Pyruvate kinase	Glyceraldehyde-P dehydrogenase	Lactate dehydrogenase
Ventricle					
Control	96 ± 11 (5)	2.05 ± 0.31 (4)	37.4 ± 6.2 (4)	2.31 ± 0.48 (4)	52 ± 14 (3)
Transgenic	120 ± 12 (5)	2.71 ± 0.77 (4)	74 ± 12[a] (5)	8.49 ± 0.42[b] (5)	69 ± 11 (5)
Soleus					
Control	214 ± 21 (5)	2.14 ± 0.37 (3)	69 ± 30 (2)		66.6 ± 4.3 (3)
Transgenic	237 ± 43 (5)	2.08 ± 0.48 (4)	111 ± 26 (5)	14.6 ± 4.1 (3)	69 ± 11 (3)
Gastrocnemius					
Control	235 ± 24 (5)	7.9 ± 2.9 (3)	251 ± 51 (3)	8.4 ± 1.0 (4)	239 ± 10 (3)
Transgenic	334 ± 47 (5)	21.3 ± 6.7 (5)	372 ± 28 (5)	16.2 ± 1.7[c] (10)	342 ± 24[c] (5)

[a] $p < 0.05$ relative to respective value in control mice.
[b] $p < 0.001$ relative to respective value in control mice.
[c] $p < 0.01$ relative to respective value in control mice.

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 contraction 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 deaminases, and myokinase are bound to myofibrillar proteins, mainly to the thin filament, and may participate in MgADP/MgATP regulation in myofibrils (Maughan and Godt, 1989). Indeed, we observed in total tissue extracts of both cardiac and skeletal muscles of transgenic mice, an increase in glycolytic enzyme activities, with no increase in total myokinase activity. It is thus possible that a fraction of these enzymes is bound to myofibrils *in vivo* and ensures local rephosphorylation of MgADP.

When PCr was omitted, it was clear that calcium sensitivity of control cardiac fibers was increased. Such a result was already obtained in rat heart (Ventura-Clapier *et al.*, 1987a) and is due to cross-bridge slowing and cooperative interaction between attached cross-bridges. Surprisingly, such an increased calcium sensitivity was not observed in soleus, gastrocnemius, or ventricular muscles of transgenic mice, and a similar force/calcium relationship was observed in control and transgenic muscles, suggesting that another mechanism compensated for the increased calcium sensitivity following changes in the local ATP/ADP ratio. Calcium sensitivity is determined by the binding of calcium to troponin C as well as by interactions between the other constituents of the thin filament. Calcium sensitivity of cardiac or skeletal muscle is developmentally regulated, and the role of troponin T isoforms is often put forward to explain changes in calcium sensitivity in spite of unchanged troponin C expression (Solaro *et al.*, 1988; Nassar *et al.*, 1991; Pan and Potter, 1992). One may suggest that a phenotypic change in the proteins constitutive of the thin filament will participate in maintaining constant calcium sensitivity in these transgenic muscles. Alternatively, at least in cardiac muscle, cAMP-mediated phosphorylation of the inhibitory unit of troponin (troponin I) decreases the sensitivity of myofibrils for calcium by diminishing the Ca^{2+}-affinity of troponin C (Ray and England, 1976). Phosphorylation of troponin I has been shown to be very stable (Garvey *et al.*, 1988). It may thus be possible that myofibrils of transgenic mice exhibit an enhanced phosphorylation level of contractile proteins which would decrease calcium sensitivity and compensate for the change induced by the altered ATP/ADP ratio inside myofibrils. Unfortunately, experimental data in support of such a hypothesis are lacking.

The skeletal muscle function of mice deficient in muscle CK has been investigated previously (van Deursen *et al.*, 1993). Mice lacking M-CK have lost the ability to sustain maximal force output during short periods of high work, while apparently being adapted for endurance exercise. However, cardiac function of mutant mice has not been investigated at present. To elucidate the role of the CK system in energy metabolism, other strategies designed to reduce the activity of the CK system were used, such as feeding animals with creatine analogs. This affects the creatine kinase/phosphocreatine system at the substrate site. Alternatively, acute iodoacetamide poisoning of CK has also been used (Fossel and Hoefeler, 1987; Kupriyanov *et al.*, 1991). In these models, where the function of isolated heart was impaired, a decreased developed pressure and rate pressure product were described (Mekhfi *et al.*, 1990; Zweier *et al.*, 1991). Furthermore, impairment of diastolic function and a steeper rise in stiffness at increased afterloads in association with increased energy breakdown were observed (Kapelko *et al.*, 1988; Kupriyanov *et al.*, 1991). Even more interesting was the observation, in these models, of phenotypic conversion of fast-twitch to slow-twitch fibers in skeletal muscle together with isomyosin transitions (Moerland *et al.*, 1989) and cardiac enlargement and increased economy of contraction by a shift from the fast isoform of myosin to the slow isoform in heart (Mekhfi *et al.*, 1990). It could be concluded that CK/PCr system alterations induce contractile abnormalities and that alterations in metabolic state *per se*, may lead to changes in the expression of contractile proteins.

No obvious change in size and distribution of the three fiber type populations was observed in M-CK-deficient mice (van Deursen *et al.*, 1993). However, M-CK-deficient type 2A and 2B fibers exhibited a clear metabolic phenotype change by elaborating an intermyofibrillar mitochondrial network, with a high number of relatively large mitochondria, the potential for aerobic energy generation being increased approximately twice (Veksler *et al*, 1995) explaining improved endurance performance during low intensity exercise (van Deursen *et al.*, 1993). In addition, we showed in the companion paper that mitochondria in ventricular and soleus muscles from transgenic mice have an increased sensitivity to ADP (Veksler *et al.*, 1995). Increase in mitochondrial content in "glycolytic" muscles and increased sensitivity to ADP in "oxidative" muscles appear to represent adaptations toward increased energy turnover via the adenylate pathway.

Absence of marked isomyosin shift, either in skeletal or cardiac muscle in M-CK knocked out mice is in contrast with what was observed in rat cardiac or mice skeletal muscles following β-GPA feeding (Shoubridge *et al.*, 1985; Moerland *et al.*, 1989; Mekhfi *et al.*, 1990). In these situations, a better economy of contractile force development was achieved by a switch from fast to slower myosin isoforms. The reason for such a difference is not straightforward. Despite the absence of an isomyosin shift in mouse heart following β-GPA feeding, these hearts can potentially switch totally from fast to slow myosin

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 only when CK activity reaches a certain level which allows saturation of binding sites, and when cytosolic CK appears, CK fluxes become detectable. However, contracting muscles were still able to hydrolyze PCr which suggested that M-CK was not the only enzyme catalyzing the transfer of PCr to ATP. In myofibrils, PCr is actively used up by bound CK; this has been shown in cardiac skinned fibers by the shift of the dependence of rigor tension development toward lower ATP concentrations induced by PCr (Ventura-Clapier and Veksler, 1994, for review see Ventura-Clapier *et al.* (1994)). Although we showed that such a shift was also present in fast-twitch and slow-twitch control skeletal muscle fibers, it was absent in skeletal as well as ventricular fibers of M-CK-deficient mice. No unknown enzyme or other isoform of CK, retained after skinning, was thus present and able to use PCr and regenerate ATP inside the myofibrillar compartment. In M-CK-deficient mice, due to the absence of local rephosphorylation, ADP should accumulate in myofibrils and diffuse in the cytosol toward mitochondria to be rephosphorylated either by mitochondrial CK which could work in both directions, explaining utilization of PCr during activity, or directly through translocase and oxidative phosphorylations. This direct route for ADP is favored by a decreased K_m of mitochondrial respiration for ADP, an increased mitochondrial network, and mitochondrial activity and increased glycolytic capacities (van Deursen *et al.*, 1993; Veksler *et al.*, 1995). Thus, alterations in one side of the CK system, *i.e.* utilization site, induces adaptations in the opposite site, *i.e.* synthesis site, showing the important role of CK in coupling utilization and consumption of energy inside muscle cells (for review see Saks *et al.* (1994)).

Of primary importance to clearly understand the exact extent and limit of adaptational processes in M-CK-deficent muscles is to know the kinetics of force development and, for the heart, to which extent it will sustain normal activity and respond to adrenergic stimulation or increase in workload. A need for more classical but necessary physiological data is evident in order to infer the exact extent and limit of adaptational processes as well as the real role of the specific isoenzymes of CK in muscle cells.

The possibility of completely and selectively abolishing a given function using transgenic technology is a fantastic tool for studying the exact role of one given protein within a pathway or a function, or for life. It has been disappointing, however, since functions considered as essential for life could be suppressed without lethal or morbid consequences. However, considering the dynamic of life, more may be learned from the adaptive strategies developed during this period of high potentiality which is embryonic life, in response to such specific alterations. It should be borne in mind that these strategies may involve "exotic" pathways 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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