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Muscle Creatine Kinase-deficient Mice

II. CARDIAC AND SKELETAL MUSCLES EXHIBIT TISSUE-SPECIFIC ADAPTATION OF THE MITOCHONDRIAL FUNCTION*

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Functional properties of *in situ* mitochondria and of mitochondrial creatine kinase were studied in saponin-skinned fibers taken from normal and M-creatine kinase-deficient mice. In control animals, apparent K_m values of mitochondrial respiration for ADP in cardiac (ventricular) and slow-twitch (soleus) muscles (137 ± 16 μM and 209 ± 10 μM, respectively) were manyfold higher than that in fast-twitch (gastrocnemius) muscle (7.5 ± 0.5 μM). Creatine substantially decreased the K_m values only in cardiac and slow-twitch muscles (73 ± 11 μM and 131 ± 21 μM, respectively). As compared to control, *in situ* mitochondria in transgenic ventricular and slow-twitch muscles showed two times lower K_m values for ADP, and the presence of creatine only slightly decreased the K_m values. In mutant fast-twitch muscle, a decrease rather than increase in mitochondrial sensitivity to ADP occurred, but creatine still had no effect. Furthermore, in these muscles, relatively low oxidative capacity was considerably elevated. It is suggested that in the mutant mice, impairment of energy transport function in ventricular and slow-twitch muscles is compensated by a facilitation of adenine nucleotide transportation between mitochondria and cellular ATPases; in fast-twitch muscle, mainly energy buffering function is depressed, and that is overcome by an increase in energy-producing potential.

Intracellular integration of muscle contractile activity and energy metabolism is one of the most intriguing problems in bioenergetics. Under physiological conditions, functional requirements of muscle and cellular energy supply are well coordinated, but mechanisms for maintaining this balance are still ill-defined. A number of works indicated a very important role of creatine kinase (CK)[1] in cellular energetics of muscle (for reviews see Jacobus (1985a), Wallimann *et al.* (1992), and Saks *et al.* (1994)). This enzyme catalyzes the reversible transfer of the phosphoryl group between phosphocreatine (PCr) and ATP in the reaction: $MgADP^- + PCr^{2-} + H^+ \leftrightarrow MgATP^{2-} +$ creatine.

Muscle cells express three subunit isoforms of CK: M-, B-, and the mitochondrial one. M- and B-subunits form dimeric MM, MB, and BB isoenzymes. CK isoforms are subcellularly compartmentalized; part of them are soluble, whereas a significant amount of MM-CK is bound to intracellular structures, close to the sites of energy utilization where cellular ATPases reside. MM-isoform has been found to be attached to the myofibrillar M-band (Turner *et al.*, 1973; Wallimann *et al.*, 1977), to the sarcoplasmic reticulum (Baskin and Deamer, 1970; Levitsky *et al.*, 1978), and to the sarcolemma (Sharov *et al.*, 1977; Jockers-Wretou *et al.*, 1977).

CK isoenzymes are localized also at the sites of energy production. Part of cytosolic MM-CK, predominantly in skeletal muscle, is associated with glycolytic enzymes. The second point of interaction of CK with ATP generation is at the outer surface of the inner mitochondrial membrane, where mitochondrial CK (mi-CK) isoenzyme is situated close to the adenine nucleotide translocase.

The distribution of CK isoenzymes in muscles depends on the type of metabolism. In cardiac and slow-twitch skeletal muscle with a permanent high level of energy production and utilization, there is an elevated relative proportion of mitochondrial CK (for review see Wyss *et al.* (1992)) and a relative low proportion of cytosolic subunits (Yamashita and Yoshioka, 1991). Fast-twitch skeletal muscles, having their metabolism based mainly on glycolytic activity, have a low proportion of mitochondrial isoform and a high proportion of cytosolic CK. These differences in CK isoform distribution have given rise to hypothesis of different roles of CK system in "oxidative" and "glycolytic" tissues.

According to this hypothesis, in oxidative tissues, the major role of CK operating with PCr is to provide the energy transport system. Mi-CK is functionally coupled to the adenine nucleotide translocase so that ATP generated by oxidative phosphorylation, after transport through the inner mitochondrial membrane, is transphosphorylated to PCr. This takes place at the expense of creatine diffusing from the sites of PCr utilization, *i.e.* sites where cytosolic CK is localized. Therefore, creatine and PCr are considered to be the main molecular species participating in the energy turnover between mitochondria and cellular ATPases.

High activity of mi-CK in the oxidative tissues ensures a rapid phosphorylation of creatine in the mitochondrial compartment. This is thought to keep local ADP concentration high in the vicinity of adenine nucleotide translocase and, in such a way, to decrease the apparent K_m for ADP. Obviously, such a

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[1] The abbreviations used are: CK, creatine kinase; M-CK, muscle isoform of creatine kinase; β-GPA, β-guanidinopropionic acid; mi-CK, mitochondrial creatine kinase; PCr, phosphocreatine; MES, 4-morpholineethanesulfonic acid.

property of mi-CK seems to be very important because, as has been shown recently (Saks *et al.*, 1991, 1993), in ventricular muscle (oxidative tissue) the K_m of mitochondrial respiration for ADP *in situ* is quite high, being considerably higher than calculated values for free cytoplasmic [ADP].

In contrast, in glycolytic muscles, CK is supposed to provide mostly the "energy buffering" function. High cytosolic activity of CK localized near the sites of glycolytic production of ATP and, perhaps, functionally coupled to glycolysis, serves as a temporal buffer for high energy phosphates. This keeps ATP and ADP concentrations steady during short periods of elevated muscular contractile activity, *i.e.* during increased ATP splitting. Furthermore, under these conditions, CK reaction consumes protons so that ATP hydrolysis and the activation of glycolysis in working skeletal muscle are not followed by an intracellular acidification.

Inhibition of various sites in the intracellular CK system would give much information about the role of this system in different tissues. Unfortunately, so far there is no specific inhibitor of CK that could be applied in living animals or even in isolated tissue preparation *in vitro*. Another approach for studying the CK system is to use animals fed by a creatine analog β-guanidinopropionic acid (β-GPA) (Fitch *et al.*, 1974) which acts as a competitive inhibitor of *trans*-sarcolemmal creatine transport (Fitch *et al.*, 1974; Chevli and Fitch, 1979). However, findings with this model of substrate deficiency should be interpreted with caution. Most importantly, in spite of a considerable decrease in total creatine content in muscles of β-GPA-fed rats, PCr levels are still in the K_m range of CK and sufficient to support high energy fluxes from mitochondria to myofibrils (Jacobus, 1985b).

Very recently, mice with a homozygous null mutation for the gene-encoding M-subunit of CK were created (van Deursen and Wieringa, 1992; van Deursen *et al.*, 1993). Being completely deprived of MM- and MB-isoforms of CK, muscles of these animals express normal levels of mi-CK and have normal concentrations of free ATP, PCr, and inorganic phosphate. One can suggest that certain mechanisms of compensation for the lack of M-CK are involved leading to rearrangements of cellular energy pathways. If CK plays different roles in oxidative and glycolytic muscles, these mechanisms should be tissue-specific. Therefore, we decided to investigate the intrinsic properties of mitochondria (this study) and myofibrils (Ventura-Clapier *et al.*, 1995) *in situ* in skinned fibers taken from various muscles as well as the functional ability of bound CK of these mice. The data described in the companion paper (Ventura-Clapier *et al.*, 1995) have demonstrated that no fundamental remodelling occurs in myofibrils. The results obtained in the present study show that oxidative and glycolytic muscles have different patterns of regulation of mitochondrial respiration by ADP and different types of adaptation to the M-CK deficiency, thus giving support to the hypothesis of different roles of the CK system in these muscles.

EXPERIMENTAL PROCEDURES

M-Creatine Kinase-deficient Mice—M-CK null mutant mice were generated by gene-targeting in the 129SvE-derived ES cell line AB-1 as described previously (van Deursen and Wieringa, 1992; van Deursen *et al.*, 1993). Offspring obtained in the breeding program was genotyped by polymerase chain reaction analysis 2 weeks after birth on a routine basis.

Six control C57BL/6 and 5 transgenic mice were weighed and anesthetized with an intraperitoneal injection of ethyl carbamate according to the recommendations of the Institutional Animal Care Committee (INSERM, Paris, France). Heart, soleus (predominantly slow-twitch), gastrocnemius (predominantly fast-twitch) muscles, brain, lung, kidneys, and liver were isolated, weighed, and 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.

Enzyme Analysis—Tissue samples were minced with scissors, placed into ice-cold solution (50 mg wet weight per 1 ml) containing (mM): K_2HPO_4, 100; EGTA; 1; *N*-acetyl cysteine, 15; pH 8.7, and homogenized with an Ultra-Turrax homogenizer. Tissue homogenates were incubated for 60 min at 0 °C for complete enzyme extraction and centrifuged at 13,000 × *g* for 20 min. The supernatant was used for enzyme determination.

CK activity was determined using the coupled enzyme assay of glucose-6-phosphate dehydrogenase and hexokinase producing NADPH. NADPH production was measured spectrophotometrically at 340 nm (Gilford Spectrophotometer, Corning, NY). The CK activity was assayed in a solution containing (mM): HEPES, 30; $MgCl_2$, 5; dithiothreitol, 0.5; ADP, 1.2; PCr, 20; glucose, 20; NADP, 0.6; P^1,P^5-di(adenosine-5') pentaphosphate (to inhibit myokinase), 0.01; and 2 IU/ml glucose-6-phosphate dehydrogenase and hexokinase at a pH of 7.1 and 30 °C. Determination of citrate synthase activity was performed according to Srere (1969). Each determination was carried out in duplicate.

Isoenzyme Fractionation—Isoenzymes were determined using agarose electrophoresis (1%) performed at 200 V for 60–90 min. Individual isoenzymes were observed by incubating the gel with a staining solution-soaked paper for 30 min at room temperature. Staining solution contained (mM): MES, 22 (pH 7.4); magnesium acetate, 50; glucose, 70; *N*-acetyl cysteine, 120; ADP, 9; PCr, 120; NADP, 9; P^1,P^5-di(adenosine-5') pentaphosphate, 0.3; 9 IU/ml hexokinase and 6 IU/ml glucose-6-phosphate dehydrogenase. Isoenzyme bands were visualized by observing the fluorescence of NADPH. The samples were scanned on a densitometer, and the area under the peaks was integrated.

Functional Properties of Mitochondria and Bound Creatine Kinase and Myokinase—Respiratory parameters of the total mitochondrial population were studied *in situ* in saponin-skinned fibers using the method described earlier (Veksler *et al.*, 1987) with minor changes (Veksler and Ventura-Clapier, 1994). Thin fiber bundles (100–250 μm in diameter) were excised from ventricular and skeletal muscles. The bundles were incubated with intense shaking for 30 min in solution S (see below) containing 50 μg/ml saponin to selectively destroy the integrity of the sarcolemma. Then the bundles were transferred into solution R (see below) for 10 min to wash out adenine nucleotides and PCr. All procedures were carried out at 4 °C.

Respiratory rates were determined by a Clark electrode (Yellow Spring Instruments) in an oxygraphic cell containing 10–15 fiber bundles in 3 ml of solution R at 22 °C with continuous stirring. The solubility of oxygen was taken to be 460 ng atoms of O/ml. After measurement, the bundles were removed and dried. Respiration rates were expressed as ng atoms of O/min/mg dry weight.

Solutions S and R contained 10 mM EGTA-CaEGTA buffer (free Ca^{2+} concentration, 100 nM), 3 mM free Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol, and 20 mM imidazole. Ionic strength was adjusted to 0.16 M by addition of potassium methanesulfonate. Solution S (pH 7.0) also contained 5 mM MgATP and 15 mM PCr. Solution R (pH 7.1) contained 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/ml fatty acid free bovine serum albumin, instead of high energy phosphates.

Functional activity of mi-CK in skinned fibers was assessed by enhancement in the rate of oxygen consumption after creatine addition in the presence of submaximal ADP concentrations (Veksler *et al.*, 1987). Myokinase functional activity was evaluated by an increase in the respiration rate after addition of 1 mM AMP in the presence of 0.2 mM ATP.

To obtain ADP kinetic parameters, muscle fibers were exposed to increasing [ADP] in the presence or in the absence of creatine (20 mM). The ADP-stimulated respiration above basal oxygen consumption was plotted in order to determine the apparent K_m for ADP and V_{max}.

Statistical Analysis—The data are expressed as the mean ± S.E. A Student's *t* test was used to determine statistical difference between means. A difference was considered statistically significant when the *p* value was less than 0.05. Nonlinear fits to Michaelis-Menten kinetics were computed by a nonlinear least-squares routine.

RESULTS

Anatomical Data—The data presented in Table I show that body and organ weights of mutant mice are not significantly different from those of control animals. As a consequence, the heart weight to body weight ratio is not altered in transgenic mice. Thus, no signs of cardiac hypertrophy, congestive heart failure, or change in muscle mass could be detected.

Creatine Kinase Isoenzyme Distribution—Muscles from M-CK-deficient mice demonstrated a significant decrease in total

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.

specific CK activity (Table II). Ventricular muscle of mutants contained about one-third of the control value, whereas skeletal muscles were characterized by a dramatic drop in the CK activity. Slow-twitch red soleus muscles of M-CK-deficient mice had about 7% and fast-twitch white gastrocnemius muscle had about 1% of the respective control values. These results are well expected as it has been found that CK activity in skeletal muscle is almost uniquely represented by the cytosolic M-isoform of the enzyme. Total CK activity in brain tissue did not reveal any difference between mutant and wild-type mice.

Table II also shows CK isoenzyme distribution in various tissues. Agarose electrophoresis followed by staining for CK activity demonstrated that ventricular and skeletal muscles (as well as brain tissue) of mutant animals were completely devoid of isoenzymes containing the M-CK subunit. M-CK activity in brain of control mice was also below the threshold of detection. Very low levels of MB- and BB-isoenzymes in control muscular tissues prevented their precise quantification. However, mutant murine muscles in some cases showed the clear appearance of BB-CK activity so that it was possible to estimate the percent representation of this isoenzyme.

There were no significant differences between control and transgenic tissues in mi-CK-specific activity. Thus, M-CK deficiency was followed by neither a compensatory expression of mitochondrial isoenzyme nor a significant appearance of B-CK.

Functional Activity of mi-CK and Myokinase—Functional properties of mitochondria, mi-CK, and myokinase in muscular tissues were assessed using the saponin-skinned fiber technique. This technique allows us to study the properties of the total mitochondrial population *in situ*. Saponin selectively destroys the sarcolemma, and soluble cytoplasmic constituents including proteins are washed out, while intracellular membrane structures, myofilaments, and cytoskeleton remain intact. Fig. 1 represents typical recordings of the respiratory activity of saponin-skinned ventricular fibers taken from control and transgenic mice. The basal respiratory rate in the absence of adenine nucleotides was increased after addition of 100 μM ADP. In control preparations, addition of 20 mM creatine considerably increased the oxygen consumption rate. That means that mi-CK retains its high functional ability to supply the adenine nucleotide translocase with ADP. It is important to note that saponin-skinned fibers possess a high ATPase activity due to the presence of actomyosin complex, sarcoplasmic reticulum, and parts of sarcolemma with a Na^+-K^+ pump. Consequently, there is a powerful ADP-regenerating system inside the fibers. Nevertheless, ADP generated in the CK reaction after creatine addition stimulated the mitochondrial respiration considerably. This indicates the existence of a functional coupling between mi-CK and adenine nucleotide translocase under conditions where a diffusion barrier exists for ADP coming from the external medium or from the cellular ATPases. In such cases, the percentage enhancement in the rate of oxygen consumption could be taken as an index of functional coupling between mi-CK and adenine nucleotide translocase. As can be seen from Fig. 1, the response of respiratory activity to creatine addition in the transgenic preparation was weaker than in control.

Fig. 2 shows the averaged values of the index of CK functional coupling in two oxidative tissues. In both ventricular and soleus muscles of wild-type mice, at 100 μM ADP, creatine induced a great stimulation in the oxygen consumption rate. In gastrocnemius muscle, the stimulation of respiration by creatine was negligible (not shown). Transgenic oxidative muscles demonstrated a significantly lower extent of respiratory rate enhancement by creatine, thus indicating an alteration in the relationship between supply of mitochondria with ADP by mi-CK and by diffusion from the extramitochondrial space.

The experimental protocol described in Fig. 1 allowed us also to evaluate the absolute values of mitochondrial respiration in a given tissue at high [ADP] (1 mM) in the presence of creatine. In ventricular muscle of mutant mice, this value (81.3 ± 4.3 ng atoms/min/mg dry weight, $n = 5$) was found to be significantly ($p < 0.01$) higher than in control muscle (60.6 ± 2.3 ng atoms/min/mg dry weight, $n = 5$). In contrast, respiration rates at high [ADP] in soleus muscle, being lower than in ventricular tissue, were similar in M-CK-deficient (35.1 ± 4.2 ng atoms/min/mg dry weight, $n = 4$) and wild-type (34.5 ± 3.6 ng atoms/min/mg dry weight, $n = 4$) preparations.

We have also tested the ability of myokinase compartmentalized in saponin-skinned fibers to produce ADP for mitochondrial respiration in oxidative (ventricle) and glycolytic (gastrocnemius) muscles. (Unfortunately, myokinase functional activity was not measured in soleus muscle because not enough tissue was available). Addition of 0.2 mM ATP to the medium without adenine nucleotides induced a marked rate of oxygen consumption by skinned fibers due to ADP generated by various cellular ATPases. Further addition of 1 mM AMP leads to a production of ADP in the myokinase reaction and stimulation of the mitochondrial respiration. The degree of this stimulation was the same in control and transgenic ventricles (Fig. 2). In control gastrocnemius muscle, AMP addition only slightly increased the oxygen consumption rate, the effect being approximately 5 times smaller than in ventricular tissue. However, in transgenic gastrocnemius muscle, the percentage of AMP-stimulated respiration was significantly higher than in control, being close to the values observed in ventricular muscle.

Mitochondrial Sensitivity to ADP and Respiratory Capacity of the Muscles—Alterations in the ability of mi-CK to stimulate the mitochondrial respiration found in M-CK-deficient muscles under our conditions could be induced by several causes. First, specific activities of the enzyme could be changed. This is not the case because decreased stimulation of respiration by creatine in oxidative muscles is not accompanied by a decrease in specific activity of mi-CK (Table II). Second, changes in the degree of functional coupling between mi-CK and adenine nucleotide translocase can contribute to the alteration in creatine- and AMP-induced stimulation in the oxygen consumption rate. Third, if the sensitivity of mitochondrial respiration to ADP is

TABLE II
CK isoenzyme profile of control and M-CK-deficient mice

Values are in IU per g wet weight.

	Total CK	Mitochondrial CK	MM	MB	BB
Ventricle					
Control	343 ± 55 (5)[a]	66 ± 10 (5)	278 ± 53 (5)	—[b]	—
Transgenic	103 ± 26[c] (5)	94 ± 22 (5)	—	—	9.1 ± 5.5 (5)
Soleus					
Control	1250 ± 111 (5)	111 ± 21 (5)	1139 ± 94 (5)	—	—
Transgenic	83 ± 28[d] (5)	80 ± 36 (4)	—	—	0.7 ± 0.4 (4)
Gastrocnemius					
Control	2360 ± 274 (5)	45 ± 20 (5)	2315 ± 259 (5)	—	—
Transgenic	27 ± 5[d] (4)	27 ± 4 (3)	—	—	2.2 ± 1.1 (3)
Brain					
Control	212 ± 25 (5)	12 ± 7 (5)	—	—	200 ± 20 (5)
Transgenic	220 ± 38 (5)	14 ± 11 (5)	—	—	206 ± 28 (5)

[a] Numbers in parentheses, number of animals.
[b] —, nondetectable.
[c] $p < 0.01$ relative to control mice.
[d] $p < 0.001$ relative to control mice.

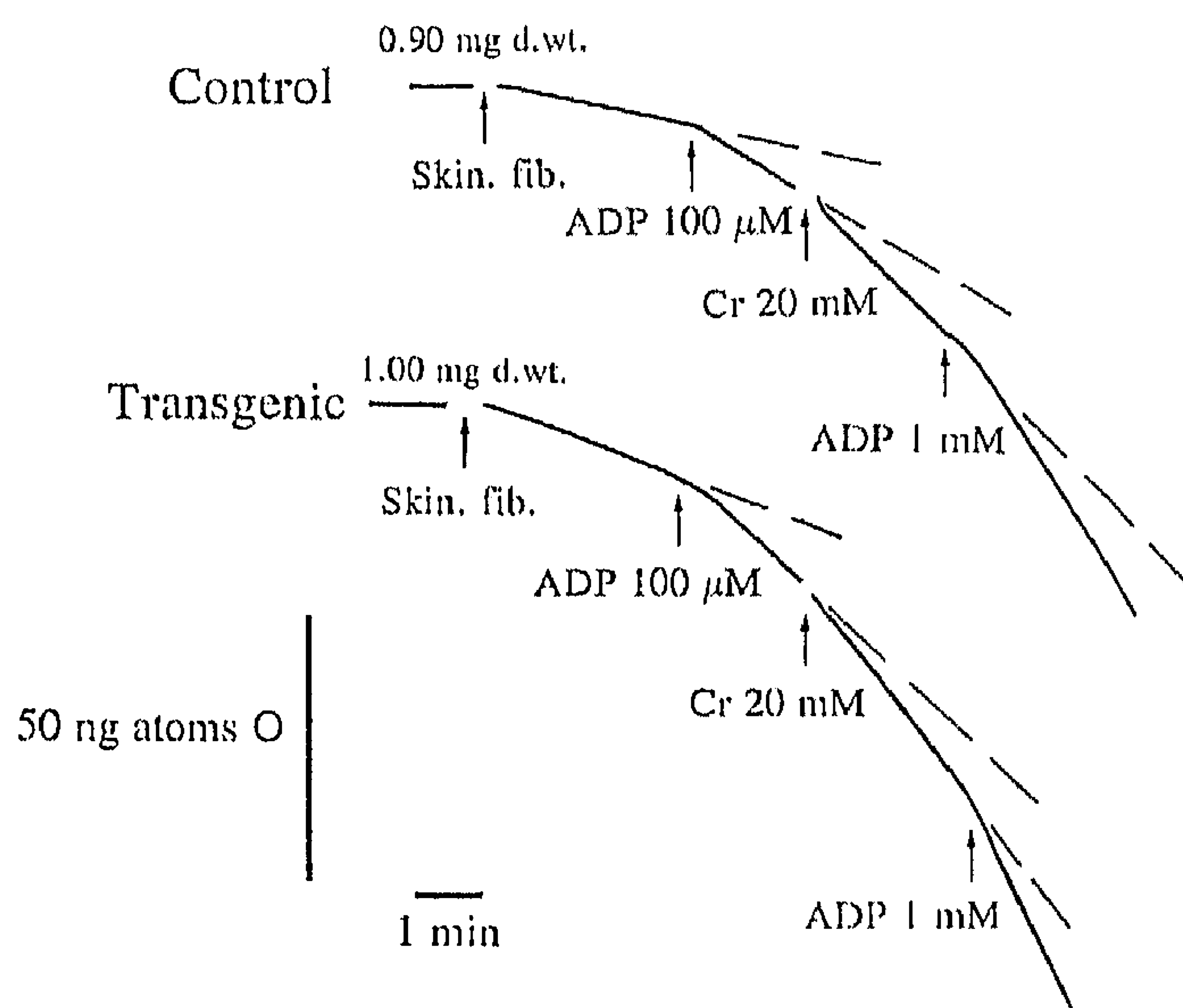

FIG. 1. **Oxygraph traces of respiratory activities of mitochondria in saponin-skinned ventricular fibers taken from control and mutant mice.** The *arrows* indicate time of additions of fibers (the quantities are expressed in mg of dry weight), ADP, and creatine. Note a weaker augmentation of the respiration rate after creatine addition in the transgenic preparation.

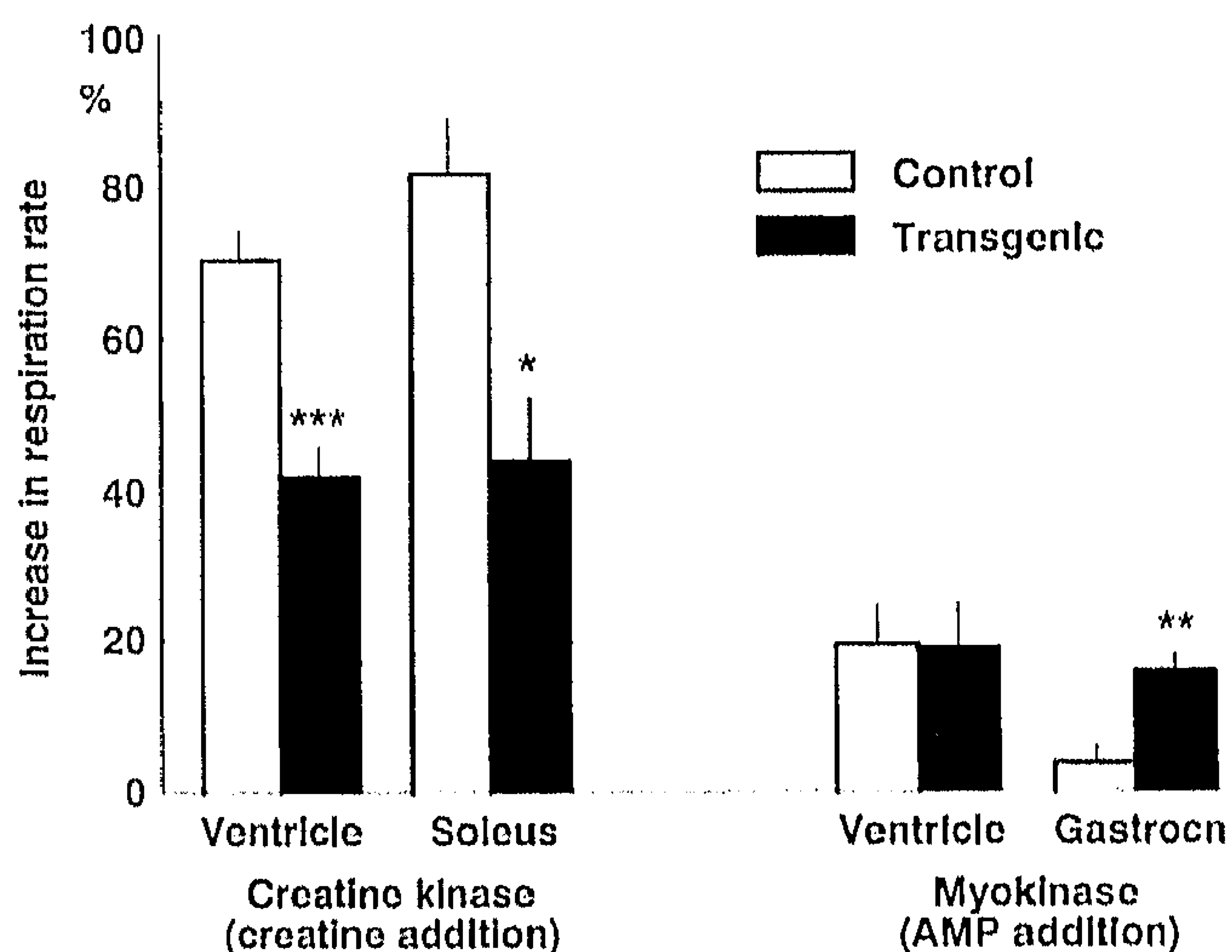

FIG. 2. **Functional activity of mi-CK and myokinase expressed as percentage of the respiration rate increase after addition of creatine or AMP respectively.** *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* respective controls.

shifted in the muscles of mutant mice, this would serve as a cause of the aforementioned effects.

To check this possibility, we studied the dependence of the mitochondrial respiration rate in saponin-skinned fibers on ADP concentration. Although the fibers possessed high ATPase activity, to keep [ADP] constant during the measurement of respiration rate at any given [ADP] in the medium, in some experiments we added an ADP-regenerating system: 20 mM glucose and 5 IU/ml hexokinase. The results were similar in the presence and in the absence of this system.

Fig. 3*a* shows the dependence of respiration on ADP concentration in representative control ventricular fibers in the presence and in the absence of creatine. The preparations were taken from the same animal. The dependence is very well fitted by the Michaelis-Menten equation. From this figure and from the double-reciprocal plot of the data (Fig. 3*b*), one can see that creatine substantially decreased the apparent K_m for ADP and, at the same time, increased the maximal respiration rate. These effects reflect the effective coupling between mi-CK and oxidative phosphorylation. The difference between the [ADP]/respiration rate curves explains the stimulation of the oxygen consumption rate by creatine at submaximal ADP concentrations.

As Fig. 3, *c* and *d*, shows, in transgenic ventricular muscle preparations (also taken from the same animal) the dependence of respiration rate on [ADP] in the absence of creatine was greatly altered. However, in the presence of creatine, the curves were very similar; as a result, apparent K_m values for ADP in the presence and in the absence of creatine are much closer than in the case of control.

Averaged values of mitochondrial kinetics are presented in Table III. In control ventricular muscle, K_m for ADP in the absence of creatine was found to be 137 ± 16 μM, a value much higher than those reported for isolated mitochondria (Chance and Williams, 1955; Bygrave and Lehninger, 1967).

Addition of creatine switches on coupled mi-CK, resulting in a more than a two times decrease of apparent K_m in control ventricular muscle (Table III). Calculated V_{max} of oxygen consumption in this muscle was higher in the presence of creatine, probably due to an accelerated production of ADP at the expense of ATP by mi-CK in the vicinity of adenine nucleotide translocase.

Mitochondria in transgenic ventricular tissue showed altered sensitivity to ADP. In the absence of creatine, K_m of

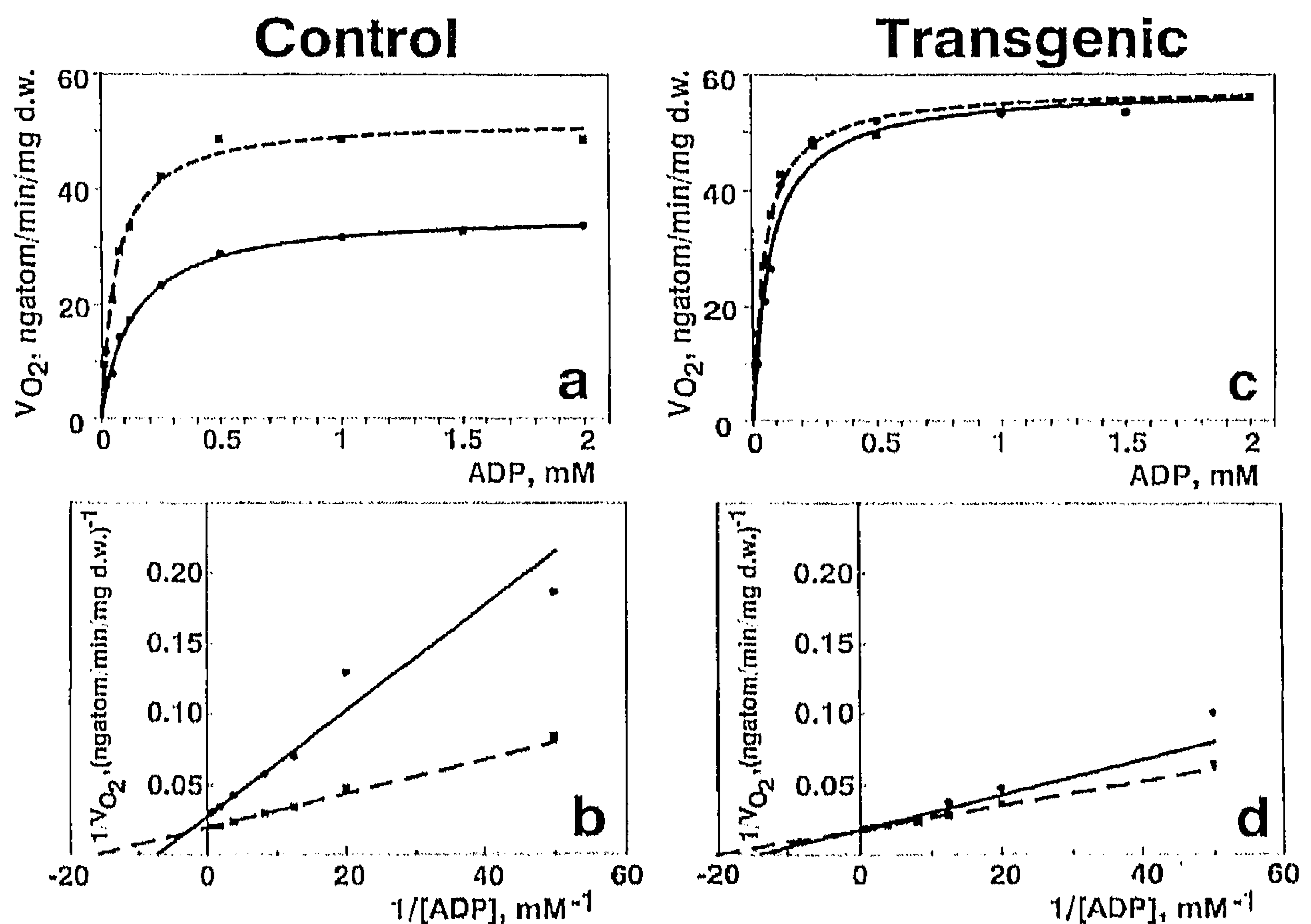

FIG. 3. **O_2 consumption rates of skinned ventricular fibers plotted as a function of ADP concentration.** Respiration of fibers taken from control (*a* and *b*) and mutant (*c* and *d*) mice was measured in the absence (*squares, solid lines*) or in the presence (*circles, broken lines*) of 20 mM creatine. Experimental data were fitted by a Michaelis-Menten equation (*a* and *c*); *b* and *d*, double reciprocal plots of $1/O_2$ consumption rates *versus* 1/[ADP] (data were derived from *a* and *c*, respectively).

TABLE III
Characteristics of in situ mitochondria in muscles of control and M-CK-deficient mice

Values are mean of 1–3 preparations from each of 5 control and 5 transgenic mice ± S.E.M.

	K_m for ADP		V_{max}		Acceptor control ratio	
		+ Creatine (20 mM)		+ Creatine (20 mM)		+ Creatine (20 mM)
	μM		ng atoms O/min/mg dry wt			
Ventricle						
Control	137 ± 16	56 ± 4[a]	52.4 ± 2.0	64.2 ± 2.4[b]	4.9 ± 0.4	5.3 ± 0.3
Transgenic	73 ± 11[c]	57 ± 5	69.1 ± 4.8[d]	67.6 ± 2.1	5.0 ± 0.7	6.2 ± 0.7
Soleus						
Control	209 ± 10	90 ± 15[a]	30.6 ± 4.0	27.4 ± 5.8	6.4 ± 0.7	4.6 ± 0.9
Transgenic	131 ± 21[d]		30.0 ± 4.2		5.4 ± 0.7	
Gastrocnemius						
Control	7.5 ± 0.5	8.8 ± 0.9	14.5 ± 1.0	13.3 ± 1.4	7.3 ± 0.8	7.3 ± 1.0
Transgenic	18.0 ± 3.4[d]	18.0 ± 2.5[c]	22.5 ± 1.9[c]	22.1 ± 1.2[c]	7.0 ± 0.3	7.0 ± 0.6

[a] $p < 0.001$ relative to respective values in the absence of creatine.
[b] $p < 0.01$ relative to respective values in the absence of creatine.
[c] $p < 0.01$ relative to respective values in control mice.
[d] $p < 0.05$ relative to respective values in control mice.

oxidative phosphorylation for ADP was about two times lower than that in control. This means that in mutant myocardium, access of extramitochondrial ADP to adenine nucleotide translocase and, possibly, export of ATP from the mitochondrial compartment were facilitated. This could contribute to the significantly higher maximal respiration rate observed in the absence of creatine, in transgenic ventricular fibers, as compared to control fibers. Creatine addition only slightly decreased the apparent K_m. Interestingly, in the presence of creatine, neither K_m for ADP nor V_{max} was different from control values in the same conditions.

These data clearly show that in M-CK-deficient ventricular muscle, the properties of the mitochondrial compartment are essentially changed so that the importance of coupled mi-CK for facilitating the ADP/ATP exchange between the mitochondrial and cytosolic compartments is diminished. The adapted mitochondria themselves or, more precisely, contact structures between the inner mitochondrial membrane and cytosol appear to be able to facilitate adenine nucleotide transportation. Qualitatively, the same data were obtained for another oxidative muscle, soleus (Table III). In the absence of creatine, skinned fibers from wild-type mice showed quite high K_m values for ADP, even higher than in ventricular muscle (209 ± 10 μM). Mi-CK was well coupled to oxidative phosphorylation because creatine significantly decreased the K_m for ADP. However, in transgenic soleus muscle, the K_m was already significantly lower in the absence of creatine, like in ventricular muscle. Thus, these results demonstrate that two types of oxidative muscles have similar low mitochondrial sensitivity to ADP in control, and the M-CK deficiency seems to be followed by similar adaptive changes with regard to K_m.

In transgenic soleus muscle, in contrast to ventricular muscle, there was no increase in the maximal oxygen consumption rate in the absence of creatine, as compared to control. In general, this muscle was characterized by lower oxidative capacity, so that the V_{max} was about 50% of the respective value for ventricular tissue.

Properties of total mitochondrial population in glycolytic, predominantly fast-twitch white muscle (gastrocnemius) were completely different from those in oxidative tissues (Table III). In control muscle, mitochondrial sensitivity to ADP was extremely high, the K_m for ADP being as low as 7.5 ± 0.5 μM, that is ≈20–25 times smaller than in oxidative muscles. Despite the presence of mi-CK, creatine did not change kinetic parameters

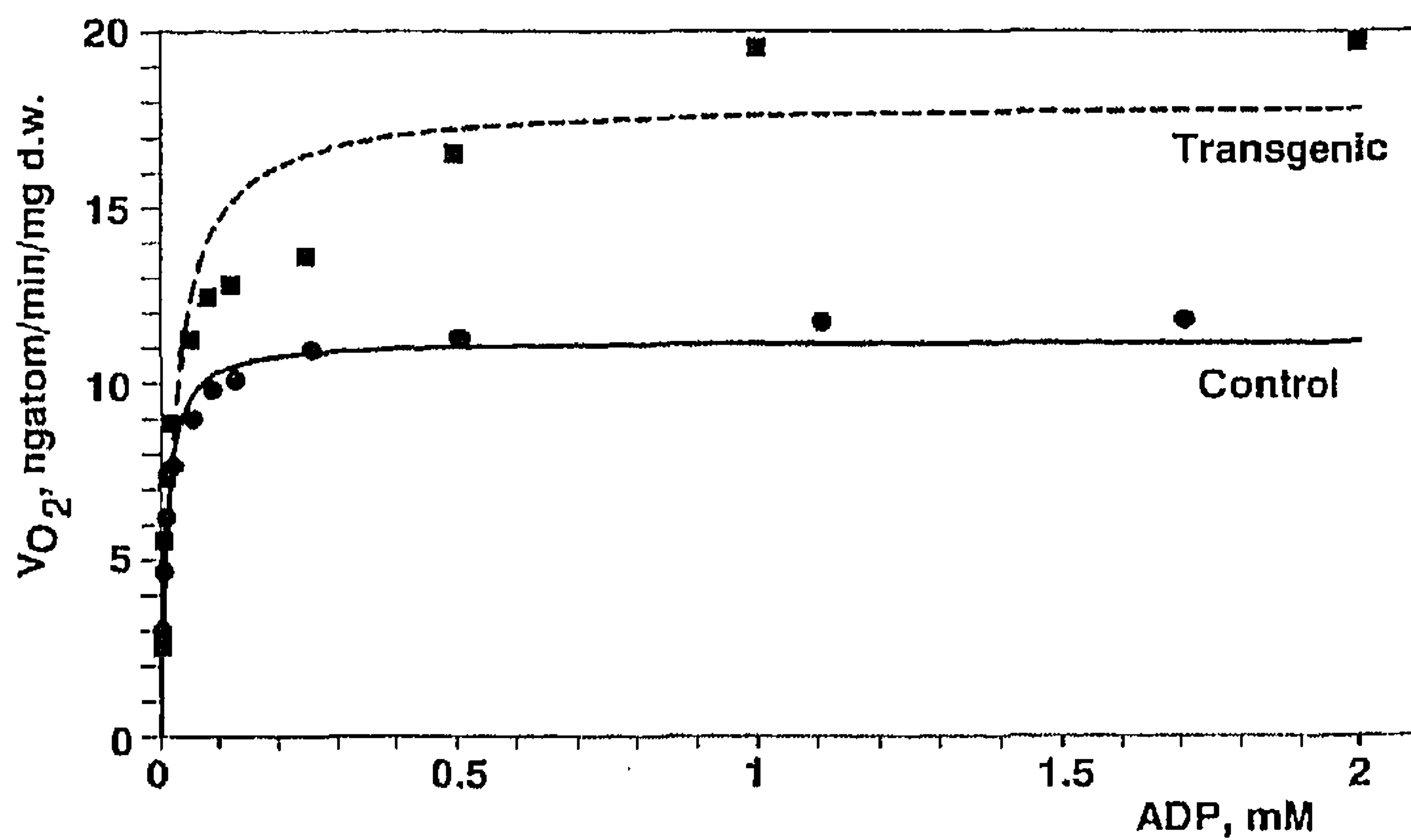

FIG. 4. **O_2 consumption rates of skinned fibers from gastrocnemius muscle plotted as a function of ADP concentrations.** Respiration of fibers taken from control (*squares*) and mutant (*circles*) mice was measured in the absence of creatine. Experimental data were fitted by a Michaelis-Menten equation for control (*solid line*) and transgenic (*broken line*) preparations.

of respiration, thus indicating either the absence of coupling between mi-CK and oxidative phosphorylation or the absence of a role for this coupling in regulation of oxidative phosphorylation in fast-twitch muscle. As expected, oxidative capacity of this muscle estimated by the maximal oxygen consumption rate was considerably lower than in oxidative muscles.

In contrast to ventricular and soleus muscles, in gastrocnemius muscle, M-CK deficiency induced a decrease rather than an increase in mitochondrial sensitivity to ADP. The apparent K_m values for ADP both in the absence and in the presence of creatine were approximately 2.5 times higher than those for control tissue. At the same time, oxidative capacity of the fast-twitch transgenic muscle was significantly increased (Table III).

Obviously, the increase in K_m for ADP as well as the rise in the maximal rate of oxygen consumption could be the result of either uniform changes in properties of total mitochondrial population or the appearance of a new population of mitochondria in the muscle. In the latter case, the total mitochondrial population would show a heterogeneity in their properties. Analysis of the dependence of respiration rate on ADP concentration gives evidences in favor of the existence of a different mitochondrial population in transgenic gastrocnemius muscle. It can be seen in Fig. 4 that the respiration of skinned gastrocnemius fibers taken from control mice is rather well-fitted by a single Michaelis-Menten curve, whereas respiration of transgenic fibers show clear deviations from hyperbolic dependence. Two groups of mitochondria seem to exist, one having a low K_m for ADP, as in control, and another one with a high K_m value. Unfortunately, due to technical reasons, there was no possibility to obtain enough experimental points to calculate these K_m values with adequate accuracy. Thus, one should realize that the apparent K_m values obtained in our experiments for transgenic gastrocnemius muscle seem to represent averaged K_m values for different existing mitochondrial populations. Apparently, the "new" population in M-CK-deficient white muscle having low sensitivity to ADP contributes to the increased respiratory capacity in the tissue.

Table III shows also values of acceptor control ratio (ratio of the V_{max} to basal respiration rate in the absence of ADP) determined for skinned fibers of various muscles. It can be seen that there were no statistically significant differences in this parameter between control and transgenic muscles. Thus, alterations in mitochondrial sensitivity to ADP found in muscles of mutant mice were not accompanied with changes in coupling between oxygen consumption and phosphorylation.

To check if the adaptation to M-CK deficiency in oxidative and glycolytic muscles is followed by alterations in quantity of respiratory units, the specific activity of citrate synthase, a marker mitochondrial matrix enzyme, was determined in extracts of ventricular and gastrocnemius muscles. Citrate synthase activity in transgenic ventricular tissue (74 ± 18 IU/g wet weight, n = 4) had a tendency to be higher than in control (39 ± 5 IU/g wet weight, n = 3) although the difference was not statistically significant. However, citrate synthase activity in mutant gastrocnemius muscle (21.3 ± 2.9 IU/g wet weight, n = 5) was significantly higher than in control muscle (6.3 ± 0.6 IU/g wet weight, n = 3), thus implying a prominent rise in oxidative potential in genetically glycolytic muscle under conditions of M-CK deficiency.

DISCUSSION

M-CK-deficient mice represent a very useful model for studying the role of the CK system in muscle tissue. In a previous article, van Deursen *et al.* (1993) have reported that resting skeletal muscles (a complex of fast- and slow-twitch muscles, gastrocnemius-plantaris-soleus) of M-CK knock out mice have normal levels of ATP and PCr. PCr could be utilized because its content declines during muscle exercise. Mutant mice show no alterations in absolute muscle force, but the performance of this muscle complex is changed. In contrast to control muscles, M-CK-deficient muscles demonstrate a drop in force after a few twitches. Furthermore, ^{31}P NMR magnetization transfer methods have shown the absence of significant fluxes through the CK reaction in mutant skeletal muscles at rest in spite of the presence of mi-CK and the ability of muscles to utilize PCr during activity.

The present study has confirmed that mutant mice are anatomically similar to the control animals. Electrophoretic analysis of mutant tissues has shown a complete absence of M-subunit of CK. However, this defect was not followed by marked alterations in internal organ macrostructure. Examination of M-CK-deficient animals has revealed no signs of cardiac hypertrophy, congestive heart failure, or alteration in muscle mass.

Knocking out the gene for M-CK subunit does not result in marked changes in other subunit expression. We have not found significant differences in mi-CK-specific activity in muscle and brain tissues between mutant and control animals. Interestingly, muscles of wild-type mice contained very low levels of BB- and MB-isoenzymes, so that quantification of these forms was not possible by electrophoresis followed by CK-specific staining. Zymograms of ventricular and gastrocnemius homogenates of murine muscles obtained by van Deursen *et al.* (1993) also show the absence of clear traces of BB-isoenzyme. In contrast, ventricular (Popovich *et al.*, 1989; Awaji *et al.*, 1990; Savabi and Kirsch, 1991; Field *et al.*, 1994), as well as skeletal (Yamashita and Yoshioka, 1991), muscles of another rodent species, rat, contain marked activities of BB- and MB-isoforms. Thus, the distribution of the CK isoenzymes varies even between close mammalian species.

Some preparations from mutant muscles in our study revealed measurable quantities of the BB-form. Accordingly, a slight increase of BB-CK in ventricular tissue of M-CK-deficient mice was reported earlier (van Deursen *et al.*, 1993). However, these changes were too weak to speculate on their significance.

The most important findings of this study are that mitochondrial sensitivities to ADP are completely different between oxidative and glycolytic muscles of wild-type mice and change in opposite directions under conditions of M-CK deficiency. The K_m values of oxidative phosphorylation *in situ* for ADP in control ventricular and soleus muscles are much higher than

K_m values obtained in isolated mitochondrial preparations. Other authors (Seppet *et al.*, 1991; Saks *et al.*, 1991, 1993; Clark *et al.*, 1994) using skinned ventricular fibers or cardiomyocytes to study respiration of mitochondria also found rather high K_m values for ADP (100–300 μM). In contrast, isolated cardiac mitochondria showed severalfold lower apparent K_m values, about 20–30 μM (Chance and Williams, 1955; Bygrave and Lehninger, 1967). It is very important to understand the reason for this discrepancy, because the apparent K_m of oxidative phosphorylation for ADP is one of the basic parameters in the theory of regulation of cellular energy metabolism. It was found that disruption of the outer mitochondrial membrane in skinned cardiac fibers by an osmotic shock dramatically decreased the K_m (Saks *et al.*, 1993). Analyzing these results, Saks *et al.* (1993) have suggested that in mitochondria *in situ*, the outer mitochondrial membrane restricts free ADP diffusion from the extramitochondrial space and thus leads to an increase in the apparent K_m for ADP. The authors have proposed that some unknown factor controls the permeability of the outer membrane to ADP, and a procedure of mitochondrial isolation eliminates the regulatory role of this factor. It is possible also that the reduced ADP diffusion in nonisolated mitochondria of oxidative muscles is the consequence of a high local concentration of macromolecules in the vicinity of the mitochondrial compartment *in situ*. In agreement with this latter view are results of Gellerich *et al.* (1993) showing an increase in resistance of outer membrane to adenine nucleotides due to elevated oncotic pressure.

Whatever the cause for the low sensitivity of *in situ* mitochondria to ADP, at physiological ADP concentration in ventricular and slow-twitch muscles, the functional coupling of mi-CK with oxidative phosphorylation is a powerful and important mechanism of regulation of the ADP/ATP ratio in the vicinity of the adenine nucleotide translocase. Our data show that working mi-CK is able to significantly decrease the K_m values for ADP making them close to the cytosolic range of [ADP]. However, this function of mi-CK may be disturbed if the PCr/creatine transport system is impaired. If cytosolic M-CK is switched off, as is the case in M-CK knocking out, one end of the "PCr shuttle" is eliminated and the transport system does not work. Under these conditions, ADP rather than creatine should play the main role of diffusible phosphate acceptor in the cells. However, ADP would be able to play this role only if the sensitivity of mitochondria to ADP is higher than in normal cells. This is exactly what we have observed in oxidative fibers of M-CK-deficient mice, where the apparent K_m values for ADP were substantially lower than those in wild-type mice. Such an adaptation, at least in the heart, does not seem to involve alterations in translocase properties because the mitochondrial sensitivity to ADP in the presence of creatine is the same in mutant and control mice.

One should point out that as far as a high K_m value is a prerequisite for evidencing the coupling between the translocase and mi-CK, it is difficult to make a clear conclusion about the degree of this coupling in mutant mitochondria. It might be possible that the coupling is weaker in mutant oxidative muscles as compared to control ones.

Our data are in very good accord with those recently obtained by Clark *et al.* (1994) in rats fed by a creatine analogue, β-GPA. Such a diet reduces the cellular levels of creatine and PCr, affecting the PCr/creatine transport system at its substrate site. These authors have found a significant (3-fold) decrease in apparent K_m for ADP in skinned ventricular fibers of β-GPA-fed rats, as compared with controls.

It seems likely that in cells with an impaired PCr/creatine transport system the diffusion barrier between adenine nucleotide translocase and the extramitochondrial space becomes more permeable not only for import of ADP but also for export of ATP and, probably, for PCr as well (van Deursen *et al.*, 1993; Wallimann, 1994). M-CK-deficient muscles are able to utilize PCr during exercise, obviously, in the reaction catalyzed either by CK or some unknown enzyme which is able to metabolize PCr. Both MM- and MB-isoforms are absent, BB-isoform activity is extremely low. At least in the myofibrillar compartment of mutant muscle cells, there is no enzyme capable of using PCr (Ventura-Clapier *et al.*, 1995). Therefore, the only enzyme that can catalyze PCr degradation is mi-CK. Thus, one has to hypothesize that communications between mi-CK and its cytosolic substrates and products are somehow facilitated.

The underlying mechanism responsible for the regulation of adenine nucleotide exchange *in situ* between translocase and the extramitochondrial space in oxidative muscles is currently unclear. The data obtained in the present study and by Clark *et al.* (1994) indicate that in M-CK-deficient mice and in β-GPA-fed rats, the hypothetical factor restricting the adenine nucleotide exchange is either absent or switched off. Thus, interesting studies can be conducted by comparing the structural and functional properties of the mitochondrial compartment in these experimental models and in normal animals to identify this factor and to provide insight into the problem of adenine nucleotide exchange between the mitochondrial compartment and the cytosol.

Our data have provided evidence that regulation of mitochondrial respiration by ADP in a fast-twitch, glycolytic gastrocnemius muscle has properties completely different from those in oxidative muscles. The apparent K_m of respiration for ADP is 20–25 times lower in gastrocnemius muscles than in oxidative tissues, thus indicating the absence of a diffusion barrier for adenine nucleotides between translocase and the extramitochondrial space. The difference in K_m values could not result from higher diffusional distance between the extracellular space and mitochondria in soleus because slow oxidative muscles have been shown to have a much greater mitochondrial volume density in the subsarcolemmal area, in contrast to glycolytic muscles (Philippi and Sillau, 1994). Moreover, our data show that the K_m for ADP measured in skinned preparations does not depend on cell diameters because fibers in mouse soleus muscle are apparently not larger than those in gastrocnemius (see Fig. 2 in van Deursen *et al.* (1993)). This means that the diffusion barrier for adenine nucleotides in oxidative cells seems to be located near the boundary of mitochondrial compartment rather than in the bulk of cytosol.

Mi-CK has no influence on the ADP-stimulated respiration in gastrocnemius muscle. Therefore, functional coupling between mi-CK and translocase in fast-twitch muscle mitochondria appears to be absent. Obviously, these mitochondria do not need coupling because their sensitivity to ADP is already very high. Possibly, in this type of muscles, mi-CK plays the same role as cytosolic MM-CK does, to balance the PCr/ATP ratio, thus providing an intracellular temporal energy buffer.

Different patterns of mitochondrial sensitivity to ADP in oxidative and glycolytic muscles found in the present study are in good agreement with the hypothesis of muscle type specificity of the control of cellular respiration. In a recent work, Kushmerick *et al.* (1992) have reported the dependence of oxygen consumption rate on calculated [ADP] in slow- and fast-twitch muscle. The authors have concluded that in fast-twitch muscle (where, as the data of our work imply, the compartmentation of adenine nucleotides is either absent or negligible) but not in slow-twitch muscle, oxygen consumption rates could be explained by a feedback control of cellular respiration by cytosolic [ADP]. Evidently, in fast-twitch muscle, actual [ADP] near

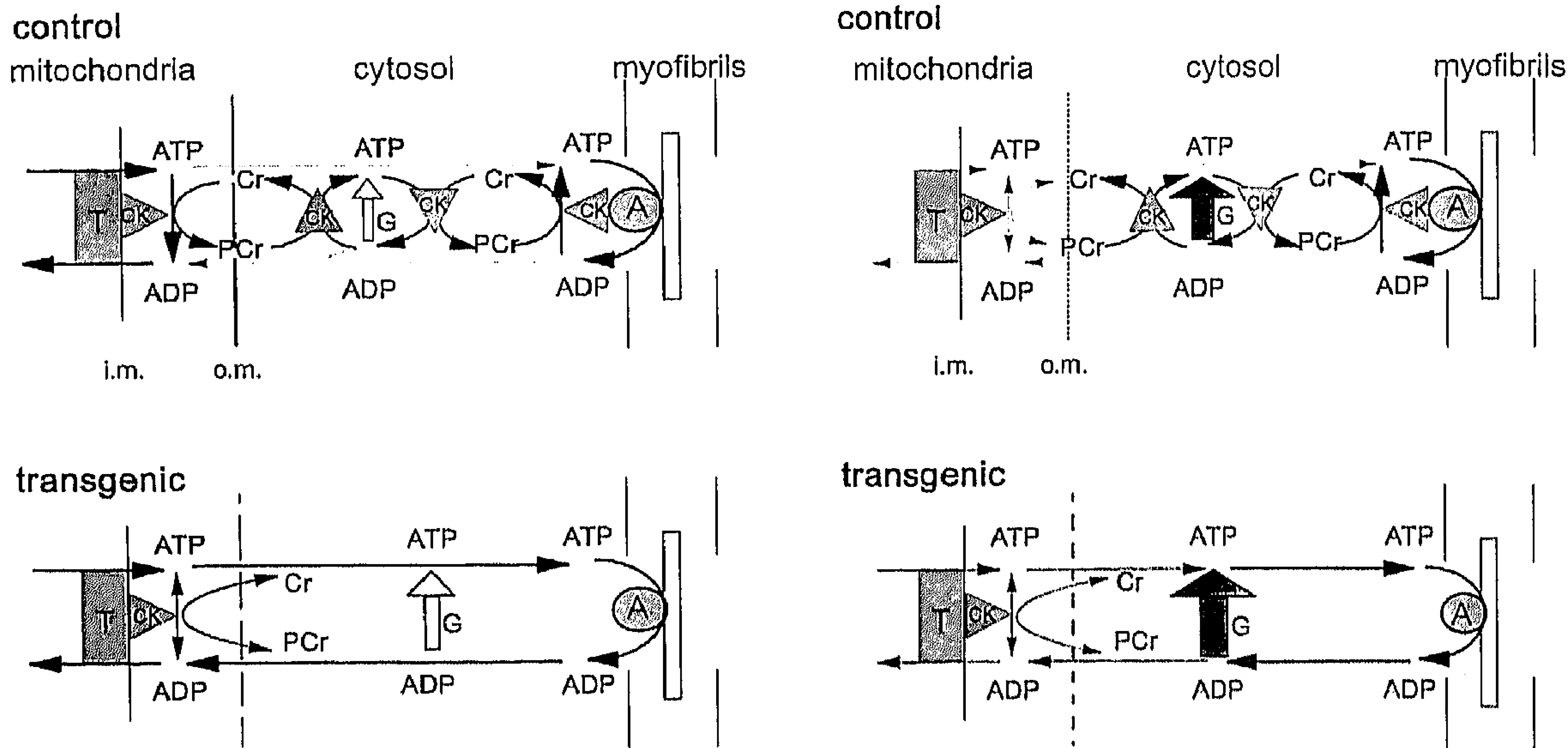

FIG. 5. **Model of main energy pathways in oxidative and glycolytic muscles of control and mutant animals.** In oxidative muscle, mitochondrial CK is functionally coupled to the translocase (*T*) on the inner mitochondrial membrane (*i.m.*) so that most of the ATP transported from the mitochondrial matrix enter the CK reaction and is transphosphorylated into PCr leaving the mitochondrial compartment. The permeability of the outer mitochondrial membrane (*o.m.*) for adenine nucleotides is quite low, and this results in high K_m of respiration for ADP. Creatine (*Cr*) imported through the outer membrane decreases the apparent K_m by increasing the local ADP concentration. In the cytosol, a relatively weak glycolytic (*G*) pathway produces ATP which is equilibrated with the PCr pool by the soluble CK. At the sites of energy utilization (*myofibrils*), ATPase (*A*) preferentially uses ATP produced at the expense of PCr in the reaction catalyzed by bound MM-CK. In the mutant oxidative muscle, due to the absence of soluble and bound MM-CK, the role of the adenylate pathway between the mitochondria and energy-consuming sites is increased. The permeability of the outer membrane for adenine nucleotides is elevated, and this is followed by a decrease in K_m of respiration for ADP. Because the diffusion barrier for ATP and ADP between extra- and intramitochondrial compartments is weaker, the muscles are able to utilize ATP produced at the expense of PCr in the CK reaction catalyzed by the mitochondrial isoenzyme. The absence of the energy-buffering function mediated in normal muscles by the soluble CK is compensated by some elevation in oxidative and glycolytic capacities. In glycolytic muscle, the contribution of oxidative phosphorylation to the ATP production is relatively small compared to glycolysis. The respiration could be controlled by cytosolic [ADP] because the adenine nucleotides are able to easily pass the barrier between extra- and intramitochondrial compartments. A high pool of PCr freely equilibrated with ATP by soluble CK can serve as an energy buffer. M-CK deficiency limits the utilization of this buffer in energy-consuming compartments. To compensate this, glycolytic and predominantly oxidative capacities increase. The muscle acquires some properties of slow-twitch skeletal muscle (and/or the proportion of slow-twitch fibers increases). The augmentation of the oxidative potential is followed by an elevation of K_m for ADP, a characteristic feature of mitochondria in oxidative muscle.

translocase appears to be close to that in cytosol calculated from data of NMR experiments.

Under conditions of M-CK deficiency, the properties of mitochondria in gastrocnemius muscle are altered. The apparent K_m for ADP increases, but a detailed analysis of the [ADP]-respiration rate relationship shows that this relationship is not well fitted by the Michaelis-Menten equation so that the determined value of the K_m probably reflects an averaged K_m value for different mitochondrial populations having different properties. One may suggest the appearance of a mitochondrial population with a high K_m for ADP, like in oxidative muscle. Furthermore, M-CK-deficient glycolytic gastrocnemius muscle increases its oxidative capacity substantially. The maximal oxygen consumption rate per unit of tissue weight rises by 50%. This could be explained by an increase in mitochondrial content. Earlier, the electron microscopy revealed an expanded mitochondrial compartment in this muscle (van Deursen *et al.*, 1993). Besides, the specific activity of citrate synthase, a marker mitochondrial enzyme, increases significantly in gastrocnemius muscle (van Deursen *et al.* (1993) and the present study).

It is interesting to note that the increase in mitochondrial content in mutant fast-twitch muscle is not accompanied by an enhancement of mi-CK activity. However, we have found alterations in mitochondrial myokinase functional activity; being very weak in control gastrocnemius muscle, the functional activity of this enzyme increased in M-CK deficiency and became close to that in oxidative ventricular fibers.

Our findings show that the adaptive strategy in white muscle seems to be directed toward appearance of some properties of red, oxidative fibers. Similar alterations were observed in fast-twitch muscle of β-GPA-fed rats where activities of aerobic enzymes (Shoubridge *et al.*, 1985) as well as mitochondrial ATP synthesis rate (Freyssenet *et al.*, 1994) were found to increase by 30–40%. Taken together, these observations suggest that in fast-twitch muscle, elimination of buffer function of CK/PCr system induces a compensatory augmentation of the oxidative potential. In contrast, slow-twitch skeletal muscle reveals another type of adaptation. We did not notice changes in the maximal respiratory capacity of skinned fibers from soleus muscle of mutant mice. Accordingly, the activities of aerobic enzymes were reported to be unchanged in soleus muscle of β-GPA-fed rats (Shoubridge *et al.*, 1985).

In the presence of creatine, the maximal oxygen consumption rate calculated from the ADP kinetics experiments was similar in mutant and control ventricular fibers (67.6 and 64.2 ng atoms/min/mg dry weight, respectively). However, respiration in the presence of high [ADP] just after addition of a low quantity of ADP and creatine (Fig. 1) was significantly higher in M-CK-deficient than in control fibers (81.3 *versus* 60.6 ng atoms/min/mg dry weight). The only difference between these two protocols is that the second one is considerably shorter. It seems likely that the adapted mitochondrial populations of mutant ventricular muscle have increased oxidative capacity,

probably due to the appearance of new mitochondria; however, they are not able to maintain high activity for a relatively long time. Some increase in citrate synthase-specific activity found in ventricular mutant muscle is of borderline significance, and this does not allow us to make any clear conclusion about mitochondrial content in M-CK-deficient ventricle.

In summary, our data show that knocking out the M-subunit of CK results in completely different patterns of adaptations in oxidative and glycolytic muscles (Fig. 5). The results obtained are consistent with the hypothesis of different roles of CK in different muscle types. Mitochondria in ventricular and soleus muscles normally have low sensitivity to ADP, and mi-CK functionally coupled with oxidative phosphorylation in the PCr/creatine pathway regulates adenine nucleotide levels in the mitochondrial compartment. It is tempting to speculate that M-CK deficiency and, consequently, an impairment of one end of the pathway result in alterations in ADP compartmentation, the role of ADP as transportable phosphate acceptor increases, and, to match these conditions, mitochondrial sensitivity to ADP rises. It appears that diffusion barriers between the mitochondrial compartment including mi-CK and extramitochondrial space become weaker, thus enabling mi-CK to serve the cytosolic compartment. This could explain the utilization of PCr by muscles of M-CK-deficient mice during exercise (van Deursen *et al.*, 1993). In normal glycolytic muscle, adenine nucleotide compartmentation is negligible, mitochondrial sensitivity to ADP is very high, and respiration is controlled by cytosolic [ADP]. Fast-twitch muscle of mutant mice is deprived of enzyme controlling utilization of a powerful energy resource (PCr), and, to compensate this potential energy deficiency, the adaptation is directed toward enhancing the oxidative capacity of the muscle. Further studies might reveal the mechanisms responsible for such different patterns of adaptation as well as identify the factor(s) controlling mitochondrial accessibility for ADP.

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