Impaired voluntary running capacity of creatine kinase-deficient mice

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The creatine kinase system (CK) is important for energy delivery in skeletal and cardiac muscles. The two main isoforms of this enzyme, cytosolic MM-CK and mitochondrial mi-CK, are expressed in a developmental and muscle-type specific manner. Mice deficient in one or both of these isoforms are viable and fertile but exhibit profound functional, metabolic and structural muscle remodelling that primarily affects fast skeletal muscles, which show an increased contribution of oxidative metabolism to contractile function. However, the consequences of these alterations in terms of physical capabilities have not yet been characterized. Consequently, we compared the voluntary exercise capacity of 9-month-old male wild-type (WT), M-CK knockout (M-CK$^{-/-}$), and M-CK and mi-CK double knockout (CK$^{-/-}$) mice, using cages equipped with running wheels. Exercise performance, calculated by total distance covered and by work done during the training period, was more than 10-fold lower in CK$^{-/-}$ mice than controls, with M-CK$^{-/-}$ mice exhibiting intermediate performance. Similarly, the mean distance run per activation was lower in M-CK$^{-/-}$ and even lower in CK$^{-/-}$ mice. However, the maximal running speed (V_{max}) was lower only for CK$^{-/-}$ mice. This was accompanied by severe skeletal muscle mass decrease in CK$^{-/-}$ mice, with signs of histological damage that included enlarged interstitial areas, aggregations of mononuclear cells in the interstitium, heterogeneity of myofibre size and the presence of very small fibres. No overt sign of cardiac dysfunction was observed by magnetic resonance imaging during dobutamine stimulation. These results show that metabolic failure induced by CK deficiency profoundly affects the ability of mice to engage in chronic bouts of endurance running exercise and that this decrease in performance is also associated with muscle wasting.

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The creatine kinase (CK) enzyme system is an important energy delivering mechanism in skeletal and cardiac muscle. CK catalyses the reversible transfer of a high-energy phosphoryl group between ADP and creatine:

phosphocreatine (PCr) + MgADP + H^+ ⇌ creatine (Cr) + MgATP

Mammalian cells express four CK subunits encoded by four different genes, the M, the B (both forming homo or hetero dimers MM, MB, BB) and two mitochondrial forms (Wallimann *et al.* 1992). The ubiquitous mitochondrial isoform (mi-CKu) is mainly expressed in brain and smooth muscle cells while the sarcomeric mitochondrial isoform (mi-CKs) is specific for skeletal and cardiac muscles. The expression of the different isoenzymes is tissue specific and developmentally regulated. Striated muscles co-express M and mi-CKs while brain co-expresses the B and mi-CKu isoforms, suggesting that the expression of these genes is highly coordinated (Payne *et al.* 1991).

The CK system is a paradigm of subcellular localized enzyme systems. In muscle cells, the different CK isoforms are compartmentalized at specific intracellular sites (mitochondria, sarcoplasmic reticulum, sarcolemma or myofilaments), or free in the cytosol. A proportion of MM-CK is structurally associated with cardiac or skeletal myofilaments at the M-line or loosely bound to sarcomeric actin filaments (Stolz & Wallimann, 1998), where it is functionally coupled to myosin ATPase (Ventura-Clapier *et al.* 1994). The same coupling occurs with the Ca^{2+}-ATPase of the sarcoplasmic reticulum

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(Minajeva *et al.* 1996; Rossi *et al.* 1990) and the Na^+–K^+-ATPase of the sarcolemma (Saks *et al.* 1984). CK isoenzymes are also localized near the sites of energy production. Part of the MM-CK is associated with glycolytic enzymes (Kupriyanov *et al.* 1978; Dillon & Clark, 1990; Kraft *et al.* 2000). The mitochondrial isoform is bound to the external face of the inner mitochondrial membrane, close to the adenine nucleotide translocase (Wyss *et al.* 1992), favouring mitochondrial respiration and the reversible CK reaction (Kay *et al.* 2000). These functional couplings create microdomains of adenine nucleotides that favour both kinetically and thermodynamically energy production on one side (low ATP/ADP ratio in mitochondria), and energy utilization on the other (high ATP/ADP near ATPases) giving rise to localized CK fluxes (Joubert *et al.* 2002; Vendelin *et al.* 2004).

The functional and structural diversity of mammalian skeletal muscles results from the diversity of skeletal muscle fibres. These fibres vary in their morphological, biochemical and contractile properties. The high degree of molecular variability is due to the existence of multigenic families encoding contractile, metabolic and calcium-handling proteins whose expression can adapt to fluctuating functional demands. The CK system is also highly adapted to specialized muscle function and is an integral part of muscle design. Two main roles have been ascribed to the CK system in muscle cells. In the first role, PCr is an energy reservoir and the CK reaction acts as a spatio-temporal buffer of adenine nucleotides (Meyer *et al.* 1984). These properties of the CK system are of particular importance in fast twitch skeletal muscles that rely on a large and quickly mobilizable energy reserve for contraction. The second role is based on the localization of CK isoenzymes on mitochondria and near the sites of energy utilization. Mitochondrial respiration is thereby controlled by mitochondrial kinases and particularly mi-CK, to ensure rapid and efficient ATP supply. This second function predominates in the slow muscles and heart, in which energy yield strictly depends on oxidative ATP production (Ventura-Clapier *et al.* 1998).

In an attempt to assess the importance of CK-catalysed reactions in cellular energy networks, mice have been generated that are deficient in either the mitochondrial (mi-$CK^{-/-}$) or cytosolic (M-$CK^{-/-}$) or both ($CK^{-/-}$) isoenzymes of CK (Steeghs *et al.* 1997; van Deursen *et al.* 1993). These animals appear normal and viable, but their skeletal muscles lack burst activity and show reduced force development, slower relaxation and altered Ca^{2+} responses (van Deursen *et al.* 1993; Steeghs *et al.* 1997; Watchko *et al.* 1997) but increased resistance to fatigue (Dahlstedt *et al.* 2000; Gorselink *et al.* 2001*a*). In addition, multiple morphological changes have been described such as a higher content of mitochondria, especially in fast skeletal muscles, with changes in mitochondrial morphology and composition, a hyperproliferative sarcoplasmic reticulum and tubular aggregates (van Deursen *et al.* 1993; Steeghs *et al.* 1997; Novotova *et al.* 2002). No fundamental remodelling occurred in myofibrils of CK transgenic mice but the main adaptations concerned mitochondrial regulation, and bioenergetic pathways including glycolytic metabolism regulation (Veksler *et al.* 1995; Ventura-Clapier *et al.* 1995; Boehm *et al.* 2000), showing that the depressed energy buffering function is overcome by an increase in energy-producing potential. On the other hand, increases in mitochondrial volume and cyto-architectural rearrangements observed in $CK^{-/-}$ mice (Steeghs *et al.* 1998; Novotova *et al.* 2002) suggest that structural adaptive mechanisms control local pools of adenine nucleotides. These cyto-architectural alterations reflect the need for a direct functional interplay between subcellular organelles in order to catalyse direct energy and signal channelling between mitochondria and the sarcoplasmic reticulum on the one hand, and between mitochondria and myofilaments on the other (Saks *et al.* 2001; Kaasik *et al.* 2001, 2003). This cyto-architectural remodelling of $CK^{-/-}$ muscle may partly rescue contractile function and calcium homeostasis. The CK system is also part of an integrated network of complementary enzymatic pathways that serve in the maintenance of energetic homeostasis and physiological efficiency. Among these pathways, adenylate kinase, the glycolytic network, and guanylate phosphotransfer pathways can take over a significant part of the overall ATP fluxes in CK-deficient animals, leading to a redistribution of energy fluxes to limit the overall energetic deficiency (Janssen *et al.* 2003; Dzeja *et al.* 2004).

However, it remains to be established whether these systems are redundant at higher workloads. Indeed, it is probable that for higher energy demands, the different systems would be additively recruited. It seems that morphological and functional adaptations are sufficient to ensure the viability of $CK^{-/-}$ mice with a sedentary laboratory lifestyle. In an attempt to evaluate the level of physical activity of $CK^{-/-}$ animals, Steeghs *et al.* (1998) have shown that spontaneous locomotion of young mice appears identical to the wild-type. However, whether the CK system would be necessary during sustained spontaneous activity remains to be established.

The aim of this work was thus to assess the importance of the CK system in physically active animals by assessing the voluntary exercise capacity of middle-aged (9 months) wild-type, M-$CK^{-/-}$ and $CK^{-/-}$ mice in cages equipped with running wheels. Output voltage of the DC generator was monitored and allowed the calculation of daily work, distance, mean distance per run, and maximal speed. Cardiac function was assessed *in vivo* by magnetic resonance imaging (MRI). Moreover, morphological, contractile and energetic parameters of different hindlimb muscles were analysed. The results show that CK deficiency profoundly affects the ability of mice to produce

spontaneous activity and is associated with lower muscle mass and signs of muscle degeneration.

Methods

Experimental protocol

Genetically engineered mice were a kind gift from Drs B. Wieringa and F. Oerlemans (University of Nijmegen, the Netherlands). Procedures involved in the generation and genotyping of M-CK$^{-/-}$ and CK$^{-/-}$ mice have been described in detail elsewhere (van Deursen *et al.* 1993; Steeghs *et al.* 1997). Nine-month-old C57BL6 wild-type (WT, $n = 13$), M-CK$^{-/-}$ ($n = 13$) and CK$^{-/-}$ ($n = 14$) mice were divided into active and sedentary groups. Voluntary physical activity of active animals ($n = 7$ WT, $n = 7$ M-CK$^{-/-}$ and $n = 8$ CK$^{-/-}$ mice) was assessed for 8 weeks; sedentary groups ($n = 6$ WT, $n = 6$ M-CK$^{-/-}$ and $n = 6$ CK$^{-/-}$ mice) were housed during this time in cages without wheels.

Voluntary activity

For voluntary wheel-running activity, animals were housed in individual cages (Bionox, Ancy, France) equipped with running wheels placed in a dark room with 12-h periods of dark and light, and free access to food and water (De Sousa *et al.* 2002; Momken *et al.* 2004). The running-wheel system consisted of a 31.8-cm diameter wheel with a 5-cm wide running surface of 365 g maximal weight in order to minimize inertia, connected to a DC generator allowing slight loading of the wheel (25×10^{-3} N m) at mean maximal speed. Output voltage of the DC generator was recorded on a PC computer during the 8-week period. Instantaneous speed, calculated from the voltage and the resistive load on the DC generator, enabled calculation of the daily running distance and work. Dividing the daily distance by the number of times each mouse used the wheel, gave the mean distance per run. Maximal speed was averaged over 1 h. All daily parameters were averaged over each week for each animal.

Eight weeks after the beginning of the experiment, the animals from the active and sedentary groups were anaesthetized with an intraperitoneal injection of pentobarbital (0.15 mg (g body wt)$^{-1}$) and killed. Cardiac, soleus (slow twitch, oxidative), plantaris (fast twitch, mixed) and superficial part of gastrocnemius (fast twitch, glycolytic) muscles were isolated, rapidly frozen and kept at −80°C. Plantaris and soleus muscles were used for morphological experiments while gastrocnemius and soleus muscles were used for biochemical analyses. The investigation conforms to Inserm Institution guidelines defined by the European Community guiding principles in the care and use of animals and the French decree no. 87/848 of October 19, 1987.

Enzyme analysis

Tissue samples were weighed, homogenized in ice-cold buffer (50 mg wet weight ml^{-1}) containing (mM): Hepes 5 (pH 8.7), ethyleneglycol-bis (β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 1 and dithiothreitol 1, with Triton X-100 (0.1%) and incubated for 60 min at 4°C for complete enzyme extraction. The total activities of creatine kinase (CK), adenylate kinase (AK), lactate dehydrogenase (LDH), cytochrome c oxidase (COX) and citrate synthase (CS) were assayed (30°C, pH 7.5) as previously described (De Sousa *et al.* 2002). CK and LDH isoenzymes were separated using agarose (1%) gel electrophoresis performed at 250 V (for CK) and 200 V (for LDH) for 90 min. Individual isoenzymes were resolved either by incubation of the gels with a coupled enzyme system (CK), or commercial detection system (Sigma LDH reagent kit). The two homo (H_4 and M_4) and three hetero (H_3M, H_2M_2 and HM_3) tetramers of LDH were resolved. H-subunit of LDH was calculated as follows:

$$\mathrm{H-LDH} = \mathrm{LDH} = \mathrm{H_4} + {}^{3}\!/_{4}\mathrm{H_3M} + {}^{1}\!/_{2}\mathrm{H_2M_2} + {}^{1}\!/_{4}\mathrm{HM_3}.$$

Muscle myosin isoform determination and histology

Native myosin was extracted from frozen tissues as previously described (Bigard *et al.* 1998). Myosin heavy chain (MHC) isoforms were separated with polyacrylamide gel electrophoresis at a constant voltage of 90 V, for 22 h, between 2 and 4°C. Transverse sections (10-μm thick) were cut from frozen plantaris and soleus muscles of CK$^{-/-}$ and WT mice in a cryostat maintained at −20°C. Each section was mounted on a coverglass, stained with haematoxylin and eosin (H&E staining), and then examined under light microscopy. Cross-sectional areas of muscle fibres were measured semi-automatically with the aid of a computer-based image-analysis system connected to a microscope (Lucia Image 4.8, Laboratory Imaging Ltd, Praha, Czech Republic). Mean fibre area values are expressed as the total of approximately 60 fibre areas divided by the total number of fibres.

MRI of the heart *in vivo*

Seven WT and nine CK$^{-/-}$ mice were anaesthetized intraperitonealy (I.P.) with ethyl carbamate (1.3 mg (g body wt)$^{-1}$). A catheter was inserted in the abdominal cavity to allow I.P. infusion of dobutamine (1.7 mg (kg body wt)$^{-1}$). Two carbon fibres placed on the thorax allowed continuous monitoring of the electrocardiogram. Cardiac MRI was performed on a 7-T horizontal imaging spectrometer (Varian, Inova) equipped with a 120 mT m^{-1} gradient coil and a custom-made birdcage probe (internal diameter, 3.6 cm). A home-built ECG gating system was used to synchronize the MRI acquisition to the Q wave. Two types of sequence

were used. First, two series of 2-dimensional multislice proton-weighted fast gradient echo cine sequences (FLASH) were recorded in short axis contiguous slices (17–19 slices of 0.5 mm thickness) in diastole, then in systole. Measurement parameters were: time of repetition (TR), 1.5 s; echo time (TE), 2.9 ms; field of view, 35 mm × 35 mm, acquisition points, 512 × 256; resolution, 500 μm × 137 μm × 137 μm; total acquisition, duration, 30 min. The morphological parameters were wall thickness, left ventricular (LV) mass and the ejection fraction (EF); stroke volume (SV) and cardiac output were calculated from these images covering the whole heart. Second, LV dynamics were assessed in the basal state and under dobutamine-induced stress on a mid-ventricular slice using a 2-dimensional short TE CINE gradient echo sequence with T1 weighting modified from (Wiesmann *et al.* 2001; Nahrendorf *et al.* 2005). Parameters were similar to the FLASH sequence except that TR was 0.120 s. Total duration was approximately 8 min. Four successive series of images were recorded every 10 ms, with initial delay after QRS of 0, 2.5, 5 or 7.5 ms (resulting in a temporal resolution of 2.5 ms), to measure the maximal ejection ($-\mathrm{d}V/\mathrm{d}t$) and filling ($+\mathrm{d}V/\mathrm{d}t$) volumes rates and their stimulation by dobutamine. EF could also be assessed on this mid-ventricular slice and was similar to that directly measured on the whole heart. Images were processed manually with one level of zero filling. At the end of experiments, animals were killed as described above.

Statistical analysis

All data are expressed as means ± S.E.M. Two-way ANOVA was used to assess the effects of CK and voluntary activity. Two-way ANOVA with repeated measurements was used to assess the effects of training. ANOVA tests were followed by Newman–Keuls *post hoc* test where appropriate. When two groups were compared, unpaired Student's *t* test was used. Values of $P \leq 0.05$ were considered significant.

Results

Effects of CK deficiency on voluntary exercise capacity

For the three groups of animals, voluntary activity was expressed as short bursts of a few seconds of running,

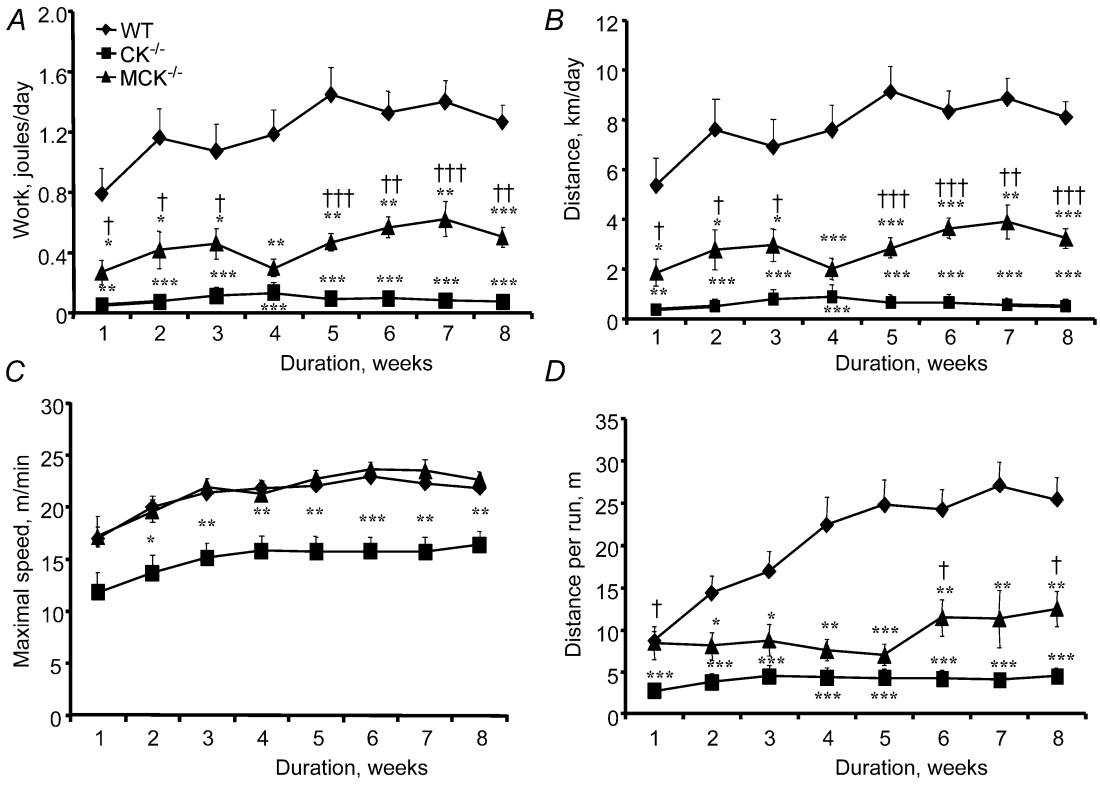

Figure 1. Weekly wheel-running parameters for wild-type (WT), cytosolic creatine kinase (M-CK$^{-/-}$) and cytosolic and mitochondrial creatine kinase (CK$^{-/-}$) deficient mice
A, work performed was much higher in WT than in CK$^{-/-}$ with intermediate values in M-CK$^{-/-}$ mice. *B*, the results were identical for the mean daily distance. *C*, the maximal running speed was significantly higher in WT and MM-CK$^{-/-}$ than in CK$^{-/-}$ mice. *D*, distance per run increased considerably over time in control mice but stayed low in CK$^{-/-}$ mice and improved slightly with time in M-CK$^{-/-}$. *,†$P < 0.05$; **,††$P < 0.01$; ***,†††$P < 0.001$. *Significantly different from control mice; †significantly different from CK$^{-/-}$ mice.

Table 1. Anatomical data

	Sed			Act			CK effect	Activity effect	Interaction
	WT	MM-CK$^{-/-}$	CK$^{-/-}$	WT	MM-CK$^{-/-}$	CK$^{-/-}$			
Body weight (BW; g)	32.9 ± 0.5	34.6 ± 2.1	34.1 ± 2.0	32.6 ± 1	37.0 ± 1.2	37.6 ± 2.0*	ns	ns	ns
Heart weight (HW; mg)	171 ± 4	227 ± 8*	194 ± 10*	216 ± 6†††	205 ± 15	201 ± 6	*	**	ns
Soleus weight (mg)	13.0 ± 0.7	7.4 ± 0.5***	8.2 ± 0.4***	15.7 ± 1.1††	8.9 ± 0.4	9.2 ± 0.7	***	**	ns
Plantaris weight (mg)	25 ± 1	22 ± 2	16 ± 1***	25 ± 2	22 ± 1	18 ± 1	***	ns	ns
HW/BW (mg g^{-1})	5.2 ± 0.1	6.0 ± 0.4*	5.7 ± 0.1*	6.6 ± 0.2††	6.1 ± 0.2	5.4 ± 0.2	*	*	*
Soleus weight/BW (mg g^{-1})	0.39 ± 0.02	0.214 ± 0.004***	0.24 ± 0.01***	0.48 ± 0.04††	0.242 ± 0.008	0.25 ± 0.02	***	**	ns
Plantaris weight/BW (mg g^{-1})	0.77 ± 0.03	0.64 ± 0.04	0.47 ± 0.04***	0.77 ± 0.07	0.61 ± 0.02	0.48 ± 0.04	***	ns	ns

Values are means ± S.E.M. ns, not significant. *$P < 0.05$; **,††$P < 0.01$; ***,†††$P < 0.001$. *CK deficient *versus* WT, †active *versus* sedentary.

always during the dark period. Figure 1 represents the weekly averaged daily work (Fig. 1*A*), running distance (Fig. 1*B*), maximal speed (Fig. 1*C*) and distance per run (Fig. 1*D*) for the 8 weeks of activity. Values for all parameters were considerably lower in CK$^{-/-}$ mice. The total running distance for all the exercise period was more than 10 times lower for CK$^{-/-}$ mice (35 ± 16 km) than for WT (433 ± 50 km, $P < 0.0001$), with an intermediate value for M-CK$^{-/-}$ mice (162 ± 24 km, $P < 0.001$). The averaged maximal speed of running was also lower in CK$^{-/-}$ (15.0 ± 1.3 m min^{-1}, $P < 0.01$) but similar in M-CK$^{-/-}$ (21.5 ± 0.6 m min^{-1}) and WT mice (21.1 ± 0.9 m min^{-1}). Mean number of runs (i.e. the number of times per day a mouse went into the wheel) was four times lower for CK$^{-/-}$ than for WT mice (96 ± 30 *versus* 409 ± 33, $P < 0.0001$) and intermediate for M-CK$^{-/-}$ mice (309 ± 18, $P < 0.05$). Mean distance per run was also five times lower (4.1 ± 0.9 m, $P < 0.001$) for CK$^{-/-}$ mice compared to the WT group (20.5 ± 2.3 m, $P < 0.01$) and again intermediate in M-CK$^{-/-}$ mice (9.4 ± 1.3 m, $P < 0.05$).

A significant improvement of work, running distance, distance per run and number of activations from the first week to the last week of activity was observed in control mice using repeated measure ANOVA ($P < 0.001$), while no effect was observed in CK$^{-/-}$ mice and intermediate results were obtained in M-CK$^{-/-}$ mice, so that a clear statistical interaction of CK ablation on training ability ($P < 0.001$) was present except for maximal speed. On the other hand, the maximal running speed significantly improved for all animals between the first and the second week of training. Altogether, although M-CK$^{-/-}$ mice showed a weak training effect, no such effect was encountered in CK$^{-/-}$ mice.

Effects of CK deficiency and voluntary exercise on anatomical parameters

Data given in Table 1 show that activity or CK deficiency had no effect on body weight in WT, M-CK$^{-/-}$ and CK$^{-/-}$ mice. A global effect of activity and CK deficiency was observed as absolute and relative heart weight increases. Activity increased cardiac weight by 26% in WT mice but not in CK-deficient mice. A strikingly lower soleus muscle mass of 40% was observed in M-CK$^{-/-}$ and CK$^{-/-}$ mice when both absolute and relative values are considered. Activity increased soleus weight in control animals by 20% but not in M-CK$^{-/-}$ or CK$^{-/-}$ animals. A 36% decrease in the plantaris muscle mass was also observed in CK$^{-/-}$ mice compared to controls. The main effect of CK deficiency was thus marked skeletal muscle weight loss and blunted cardiac and skeletal muscle hypertrophy in response to exercise.

Effects of CK deficiency and voluntary exercise on cardiac and skeletal muscle metabolic enzymes

It is now well established that the adaptive response to CK deficiency mainly concerns energy metabolism and mitochondrial function in a muscle type-specific manner. We thus assessed the energetic status of cardiac, slow and fast skeletal muscles of WT and CK$^{-/-}$ mice to evaluate whether metabolic properties are modified by exercise in these mice.

Table 2 shows that, in cardiac muscle, CK deficiency was associated with a global increase in CS activity, a marker of the Krebs cycle, while voluntary activity was without effect. In contrast, it had a significant effect on COX activity only in WT mice. A striking increase in AK activity occurred in hearts from both sedentary and active CK$^{-/-}$ mice with no effect of voluntary activity. The content of H-LDH associated with oxidative metabolism was lower in sedentary CK$^{-/-}$ mice, but increased following voluntary activity. Altogether, only slight changes in metabolic markers were found in CK$^{-/-}$ mice following exercise, suggesting that metabolic enzyme alterations would not explain the impaired exercise capacity.

In soleus muscle (Table 3), CK deficiency only led to a significant increase in the ratio of H/M LDH compared to

Table 2. Biochemical parameters of left ventricle of CK mice

	WT		$CK^{-/-}$		CK effect	Activity effect	Interaction
	Sedentary	Active	Sedentary	Active			
Metabolic enzymes							
CS (IU (g wet weight)$^{-1}$)	183 ± 21	182 ± 22	223 ± 12	224 ± 9	*	ns	ns
COX (IU (g wet weight)$^{-1}$)	420 ± 46	585 ± 33††	456 ± 30	441 ± 19*	ns	*	**
AK (IU (g wet weight)$^{-1}$)	285 ± 34	294 ± 27	401 ± 15*	437 ± 33**	***	ns	ns
CK (IU (g wet weight)$^{-1}$)	498 ± 44	441 ± 45	nd	nd	***	ns	ns
mi-CK (IU (g wet weight)$^{-1}$)	113 ± 20	97 ± 77	nd	nd	***	ns	ns
MM-CK (IU (g wet weight)$^{-1}$)	375 ± 29	324 ± 79	nd	nd	***	ns	ns
LDH (IU (g wet weight)$^{-1}$)	204 ± 15	174 ± 17	173 ± 18	210 ± 12	ns	ns	ns
H-LDH (IU (g wet weight)$^{-1}$)	122 ± 2	107 ± 8	93 ± 6*	121 ± 5†	ns	ns	**
LDH H/M subunit	1.57 ± 0.05	1.42 ± 0.12	1.27 ± 0.14	1.41 ± 0.10	ns	ns	ns

Values are means ± S.E.M. nd, not detectable; ns, not significant; CK, creatine kinase; COX, cytochrome c oxidase; CS, citrate synthase; LDH, lactate dehydrogenase; AK, adenylate kinase. *,†$P < 0.05$; **,††$P < 0.01$; ***$P < 0.001$. *deficient *versus* WT; †active *versus* sedentary. H-LDH, see Methods for calculation.

Table 3. Biochemical parameters of skeletal muscles of CK mice

	WT		$CK^{-/-}$		CK effect	Activity effect	Interaction
	Sedentary	Active	Sedentary	Active			
Soleus							
AK	408 ± 22	394 ± 21	467 ± 27	473 ± 49	ns	ns	ns
CK	1082 ± 91	1017 ± 47	nd	nd	***	ns	ns
mitochondrial CK	34 ± 4	61 ± 13	nd	nd	***	ns	ns
MM-CK	1048 ± 89	956 ± 45	nd	nd	***	ns	ns
LDH	122 ± 10	107 ± 13	110 ± 12	107 ± 18	ns	ns	ns
H-LDH subunit	122 ± 3	107 ± 12	110 ± 12	122 ± 12	ns	ns	ns
LDH H/M	0.99 ± 0.04	1.11 ± 0.12	1.42 ± 0.10*	1.21 ± 0.08	*	ns	ns
Gastrocnemius							
AK	699 ± 104	599 ± 76	1299 ± 98**	1570 ± 119***	***	ns	ns
CK	2443 ± 202	2618 ± 252	nd	nd	***	ns	ns
Mitochondrial CK	2 ± 2	13 ± 8	nd	nd	***	ns	ns
MM-CK	2641 ± 228	2605 ± 246	nd	nd	***	ns	ns
LDH	336 ± 18	262 ± 47	370 ± 51	369 ± 30	ns	ns	ns
H-LDH subunit	11 ± 2	10 ± 4	36 ± 9**	45 ± 6**	***	ns	ns
LDH H/M	0.02 ± 0.01	0.04 ± 0.01	0.11 ± 0.03**	0.14 ± 0.02**	***	ns	ns

Values are means in IU (g wet weight)$^{-1}$ ± S.E.M. nd, not detectable; ns, not significant; CK, creatine kinase; LDH, lactate dehydrogenase; AK, adenylate kinase. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *deficient *versus* control. H-LDH, see Methods for calculation.

control values, suggesting a relative increase in oxidative metabolism. Activity *per se* had no significant effect on soleus energy metabolism either in control or CK-deficient mice. There was a tendency for increased mi-CK in active WT mice, although results did not reach significance ($P = 0.09$). Markers of mitochondrial content (Fig. 2) were not different, except for a slight decrease in COX activity in active $CK^{-/-}$ mice.

On the contrary, marked changes in the metabolic phenotype were observed in gastrocnemius muscle of $CK^{-/-}$ mice. Table 3 shows a marked up-regulation of AK activity in $CK^{-/-}$ muscle as well as a more than three-fold increase in the oxidative H subunit of LDH. As already described, CS and COX activities were markedly increased in both active and sedentary $CK^{-/-}$ mice, (Fig. 2). No effect of activity *per se* was observed on the metabolic parameters of gastrocnemius muscle.

Effects of CK deficiency and voluntary exercise on myosin heavy chain expression

MHC expression was analysed in soleus, plantaris and gastrocnemius muscles (Fig. 3). In soleus of WT mice, the four MHC isoforms are expressed, while in $CK^{-/-}$ mice the fastest forms MHC-IIX and MHC-IIB are absent. A global effect of CK deficiency was observed

with an increase in MHC-IIA ($P < 0.05$), and decreases in MHC-IIX ($P < 0.001$) and MHC-IIB ($P < 0.05$). In gastrocnemius muscle, the proportion of MHC-IIX was significantly increased by CK deficiency at the expense of MHC-IIB (global effect, $P < 0.001$), with a further effect of activity only in $CK^{-/-}$ mice (global effect, $P < 0.001$) and a significant interaction between the two effects ($P < 0.05$). In plantaris muscle, which expresses only the fast MHC isoforms, MHC-IIA was absent in sedentary $CK^{-/-}$ mice and appeared after training, while MHC-IIB was lower in $CK^{-/-}$ and active WT than in WT sedentary mice. So, in contrast to the drastic changes in metabolic phenotype from glycolytic to oxidative in the fast-twitch muscle, the general effect of CK deficiency on the MHC was weak, with a slight decrease in the expression of the fastest MHC isoforms.

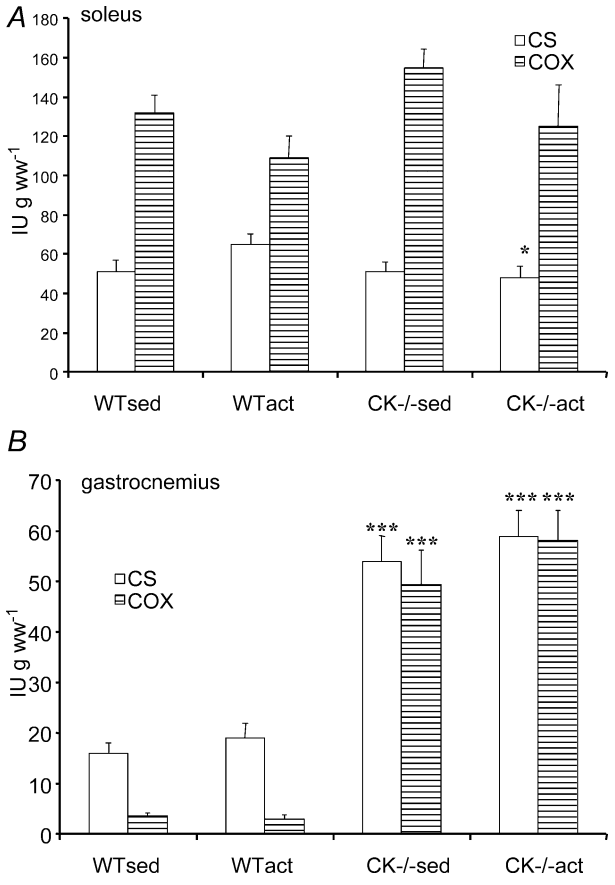

Figure 2. Mitochondrial enzyme content of soleus and gastrocnemius muscles of sedentary or active, WT or $CK^{-/-}$ mice

Citrate synthase (CS) is an enzyme of the Krebs cycle and cytochrome c oxidase (COX) is the complex IV of the respiratory chain. Small changes in mitochondrial enzyme activities are observed in soleus, both in response to CK deficiency and activity. In gastrocnemius muscle, a dramatic increase in mitochondrial enzymes is present in CK deficiency in both sedentary and active animals. *$P < 0.05$; ***$P < 0.001$. *Significantly different from control mice.

Effects of CK deficiency and voluntary exercise on soleus and plantaris muscle morphology

The histological analysis revealed restricted areas of fibre necrosis characterized by infiltration of mononucleated cells, in both soleus and plantaris muscles from $CK^{-/-}$ animals (Fig. 4*C* and *H*). These mononucleated cells were probably inflammatory cells, but the use of H&E staining did not allow the strict identification of their cell type. An enlargement of interstitial areas was frequently observed, together with endomysial infiltrates of mononucleated cells (Fig. 4*B* and *H*). The mean fibre cross-sectional area was 36% lower in soleus ($6370 \pm 212\ \mu m^2$ in WT

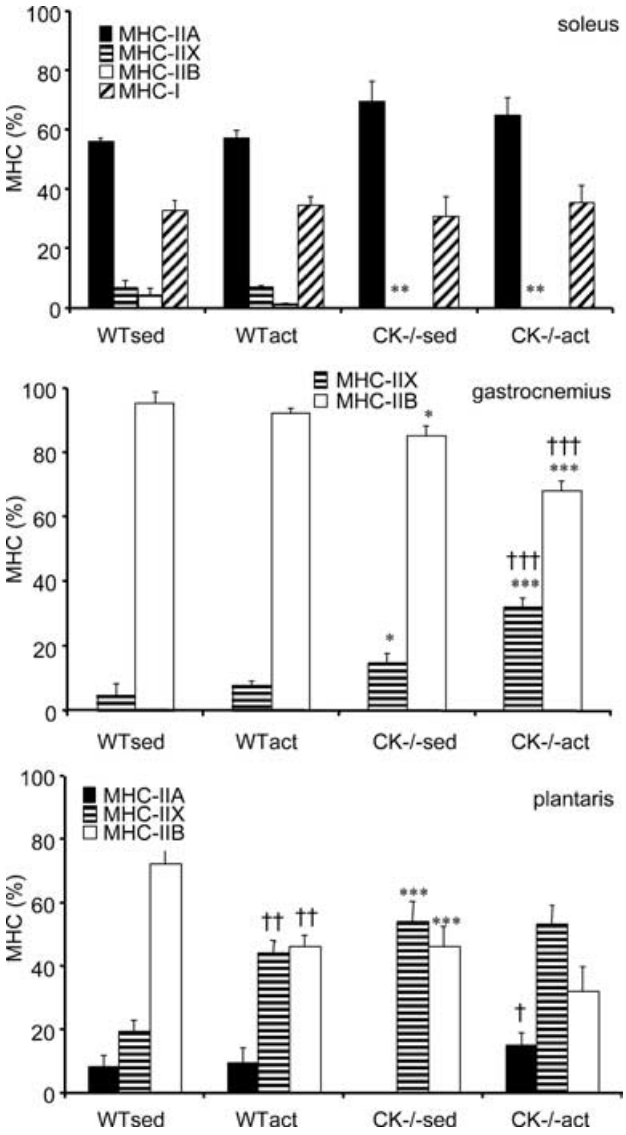

Figure 3. Myosin heavy chain (MHC) content (%) of soleus (*A*), gastrocnemius (*B*) and plantaris (*C*) muscles of sedentary or active, WT or $CK^{-/-}$ mice

*,†$P < 0.05$; **,††$P < 0.01$; ***,†††$P < 0.001$. *Significantly different WT *versus* $CK^{-/-}$ mice; †significantly different sedentary *versus* active mice.

versus $4079 \pm 259\ \mu m^2$ in $CK^{-/-}$, $P < 0.01$), and 28% lower in plantaris muscle ($7268 \pm 269\ \mu m^2$ in WT *versus* $5246 \pm 216\ \mu m^2$ in $CK^{-/-}$ mice, $P < 0.01$). Myofibre size heterogeneity was observed, with many small fibres very close to large myofibres of rounded appearance, in soleus (Fig. 4*D*) as well as in plantaris muscles. In association with these focal signs of myofibre degeneration, myofibres with internal nuclei were observed with a similar incidence in soleus and in plantaris muscles (Fig. 4*B–D*, *G* and *H*). The presence of fibres with central nuclei is usually considered as a marker of myofibre regeneration (Rosenblatt & Woods, 1992). Moreover, focal areas of connective tissue were detected in the extracellular compartment (Fig. 4*F* and *G*), and histological structures difficult to identify were occasionally observed in some cross sections (Fig. 4*B* and *C*). Connective tissue extends around myofibres, together with slight infiltrations of mononucleated cells, and induces fibrous coating (Fig. 4*B* and *H*). These histopathological changes were seen in skeletal muscles of $CK^{-/-}$ animals, in both sedentary and active mice.

Effects of CK deficiency on cardiac imaging

Table 4 compares the morphological and functional cardiac parameters of anaesthetized sedentary WT ($n = 7$) and $CK^{-/-}$ ($n = 9$) mice *in vivo* assessed by MRI. Morphological data from this group of animals revealed a 24% cardiac hypertrophy with an increase in heart weight to body weight ratio from $3.9 \pm 0.2\ mg\ g^{-1}$ to $4.8 \pm 0.1\ mg\ g^{-1}$ in $CK^{-/-}$ mice ($P < 0.01$). Both MRI-determined LV mass and LV mass/body weight ratio were 28% higher in $CK^{-/-}$ mice than in WT confirming the presence of cardiac hypertrophy. Wall thickness, stroke volume, cardiac output and ejection fraction in the basal state, were similar in WT and $CK^{-/-}$ animals. After checking on an isolated perfused heart preparation that the sensitivity to β-adrenergic stimulation was unchanged by CK deletion (data not shown), a dobutamine stress was performed in five WT and seven $CK^{-/-}$ mice. Figure 5*A* and *B* shows representative mid-ventricular images in systole and diastole in a $CK^{-/-}$ mouse, as well as the LV surface cavity–time curves used to measure the maximum LV filling and ejection rates (Fig. 5*C*) under basal and maximal contractility induced by dobutamine stress. Kinetic parameters were similar in both groups showing that CK deletion did not overtly impair dobutamine-stimulated cardiac function *in vivo*.

Discussion

This study is the first report of the voluntary exercise capacity of mice deficient in the cytosolic (M-$CK^{-/-}$) or both the cytosolic and mitochondrial ($CK^{-/-}$) isoforms of creatine kinase using voluntary wheel running. The novel findings can be summarized as follows. (1) The total distance and work covered during the training period were more than 10-fold lower for $CK^{-/-}$ mice than for controls, with M-$CK^{-/-}$ mice exhibiting intermediate performance. (2) The mean distance per activation was much lower for $CK^{-/-}$ mice than for controls, again with intermediate values for M-$CK^{-/-}$ mice. (3) The maximal speed of running was 50% lower in $CK^{-/-}$ mice only. (4) $CK^{-/-}$ mouse cardiac and skeletal muscle metabolic enzymes were not altered by activity. (5) A significant correlation was found between exercise capacity and muscle mass in CK-deficient and control mice, together with signs of severe histological damage in CK-null animals.

Both forced and voluntary exercise protocols can be used to evaluate exercise performance in laboratory animals. However, these two exercise protocols may reflect different exercise abilities as no apparent correlation could be found between treadmill performance and voluntary running performance (Lerman *et al.* 2002). Voluntary wheel running has been used for investigating the impact of

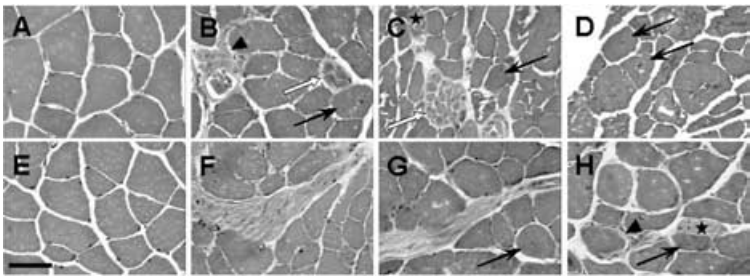

Figure 4. Light microscopy cross-sections of soleus (*A–D*) and plantaris muscles (*E–H*) taken from WT (*A* and *E*) and $CK^{-/-}$ mice (*B–D* and *F–H*)
Transverse sections (H&E staining) show enlarged interstitial areas, endomysial infiltrates of mononucleated cells (arrow heads), heterogeneity in myofibre size with small fibres close to large rounded myofibres (*D*), features of degenerating fibres (filled stars), myofibres with internal nuclei (black arrows), focal and large areas of connective tissue (*F* and *G*) and histological structures with degenerative features that are difficult to identify (white arrows). Connective tissue was shown to extend around myofibres and to induce fibrous coating (arrow heads). Scale bar, 50 μm.

Table 4. Cardiac magnetic resonance imaging of WT and CK$^{-/-}$ mice

	WT	CK$^{-/-}$
Anatomy and basal contractility	($n = 7$)	($n = 9$)
Mean LV mass (mg)	130.6 ± 8.3	163.2 ± 3.6*
Mean LV mass BW (mg g^{-1})	3.39 ± 0.17	4.35 ± 0.12**
End Diastolic LV thickness (mm)	1.33 ± 0.05	1.32 ± 0.06
Systolic LV thickness (mm)	2.45 ± 0.06	2.43 ± 0.09
Stroke volume (μl)	27.1 ± 2.7	31.6 ± 3.1
Cardiac output (ml min^{-1})	16.7 ± 2.0	21.6 ± 2.4
Heart rate (beats min^{-1})	611 ± 30	683 ± 7
Ejection fraction	0.90 ± 0.02	0.90 ± 0.02
V_{max} ejection (mm^2 ms^{-1})	-0.35 ± 0.02	-0.37 ± 0.04
V_{max} filling (mm^2 ms^{-1})	0.29 ± 0.03	0.31 ± 0.02
Contractility under dobutamine-induced stress	($n = 5$)	($n = 7$)
Heart rate (beats min^{-1})	637 ± 23	688 ± 10
Ejection fraction	0.96 ± 0.01†	0.95 ± 0.01†
V_{max} ejection (mm^2 ms^{-1})	-0.47 ± 0.03†	-0.52 ± 0.05†
V_{max} filling (mm^2 ms^{-1})	0.29 ± 0.03	0.34 ± 0.02

†Dobutamine *versus* control. *deficient *versus* WT. Dobutamine infusion, 1.7 mg kg^{-1}, I.P. BW, body weight; LV, left ventricle.

ablating a specific gene on running performance (Harrison *et al.* 2002). It allows the animals to exercise with respect to their spontaneous day and night life, intensity and pattern of exercise, while avoiding the stress inherent in treadmill running, although this results in a wide range of exercise performances by different animals. However, it cannot be taken as an assessment of the maximal exercise capacities. Although the mice used in this study were older (9 months) than those used in previous studies, the results were similar to those previously published with the same strain (C57B6) in terms of running distance and maximal speed of running (Allen *et al.* 2001; Lerman *et al.* 2002). We derived a set of parameters that reflect different aspects of physical performance. Total work (in joules), daily running distance, maximal running speed and mean distance per run significantly increased during up to 5 weeks of exercise and remained constant thereafter. Thus, the present programme of 8 weeks of voluntary wheel running led to a significant improvement in performance in WT mice. Whether this is related to a real increase in endurance capacity or reflects learning and familiarization is difficult to assess. Nevertheless, this is consistent with previous reports showing that 4 weeks of voluntary wheel running in mice are sufficient to produce specific adaptations related to endurance training in both the cardiac and skeletal muscles (Allen *et al.* 2001). By contrast, CK-deficient mice had lower exercise capacities than controls. These animals ran shorter distances, performed less work and ran less each time they used the wheels. Thus, although CK deficiency did not cause an overt phenotype in sedentary animals, it greatly impaired their physical activity. That this impairment relates to a decreased ability rather than motivation to perform exercise could be suggested from the strong correlation between voluntary exercise capacity and muscle mass described below. It is interesting that mice deficient in the cytosolic CK isoform were affected less severely than mice deficient in both cytosolic and mitochondrial CK, as was evident for most parameters after 5 weeks of running. Moreover, maximal running speed was lower only in mice deficient in both M- and mi-CK isoforms. Thus a clear additional defect is brought about when the mitochondrial isoform is also deleted, reflecting either the importance of this specific isoform for voluntary exercise capacity, and/or the lower remaining CK activity.

Exercise performance is a highly complex trait with a multivariant interaction between pulmonary, cardiovascular, psychological and morphological factors. In the case of CK deficiency, these two isoenzymes being expressed mainly in striated muscles, it can be inferred that mainly cardiac and skeletal muscle alterations are involved.

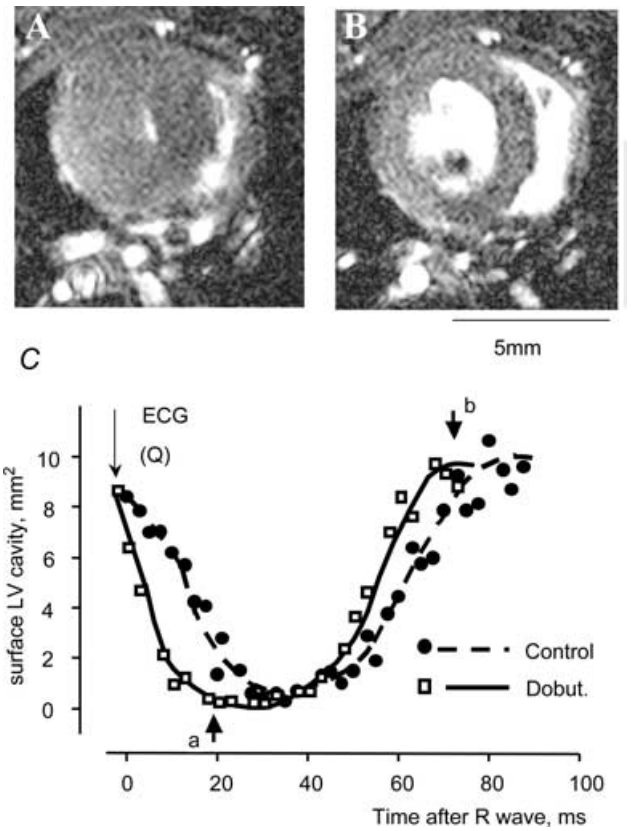

Figure 5. MRI determination of kinetics of contraction of CK$^{-/-}$ mice
Mid-ventricular image of a CK$^{-/-}$ mouse heart under dobutamine-induced stress in end systole (*A*) and in end diastole (*B*) used to measure contraction kinetics (slice thickness, 0.5 mm). *C*, LV surface–time curve of a CK$^{-/-}$ mouse at rest and under dobutamine-induced stress: the slice area of the LV cavity is plotted as a function of time after the R wave, allowing calculation of the maximal rates of ventricular filling and ejection.

Cardiac adaptations and function

CK deficiency caused a significant 20–30% cardiac hypertrophy, as previously reported (Boehm *et al.* 2000; Gustafson & VanBeek, 2002), associated with LV dilatation (Nahrendorf *et al.* 2005). In addition to increased cardiac CS content in $CK^{-/-}$ mice, this reflects an adaptive mechanism to CK deficiency. By contrast, voluntary running exercise was sufficient to induce physiological cardiac hypertrophy in WT mice but not in $CK^{-/-}$ mice. While the running activity of these mice could have been too low to induce a significant stress leading to hypertrophy, the inability of CK-deficient mice to undergo further hypertrophy under stress, however, cannot be ruled out (Nahrendorf *et al.* 2005).

Exercise capacity also depends on cardiac function. Cardiac output has been positively correlated with treadmill performance across 11 inbred rat strains (Barbato *et al.* 1998). However, no significant correlation between echocardiographic data at rest and exercise performance have been found in mice of different strains (Lerman *et al.* 2002), evidencing the need to assess cardiac function under conditions of stress. This was achieved in this study by infusion of dobutamine. We used MRI to assess cardiac function *in vivo*. Again a 20–30% cardiac hypertrophy was detected in $CK^{-/-}$ mice and in these conditions the cardiac function of $CK^{-/-}$ mice was similar to WT mice basally and following catecholamine-induced stimulation. This is consistent with studies using isolated hearts from M-$CK^{-/-}$ or $CK^{-/-}$ animals, subjected to increased calcium concentration (Saupe *et al.* 1998) or increased stimulation frequency (VanDorsten *et al.* 1998), while a cardiac phenotype could be clearly unmasked following acute stress conditions induced by ischaemia–reperfusion (Spindler *et al.* 2004). In a previous *in vivo* study, a decreased responsiveness to catecholamines was detected in $CK^{-/-}$ mice using echocardiographic characterization of anaesthetized animals (Crozatier *et al.* 2002). In a recent *in vivo* study using MRI, Nahrendorf *et al.* (2005) showed significant LV hypertrophy and dilatation in $CK^{-/-}$ mice allowing similar basal ejection fraction. Thus, the nearly preserved *in vivo* cardiac function of CK-deficient animals, underlines the effectiveness of compensatory energy transfer mechanisms in cardiac cells (Saupe *et al.* 1998; Kaasik *et al.* 2001; Bonz *et al.* 2002), and precludes the possibility that cardiac defects may be the primary factor responsible for decreased voluntary exercise capacity in CK-deficient mice.

Contractile and metabolic muscle adaptations

The muscle phenotype consists of two main components, a contractile and a metabolic one, by which different muscle types are adapted to different functions, and that may or may not be co-regulated in response to external factors such as exercise, load or hormonal interventions. As already reported (Steeghs *et al.* 1998), despite heavy metabolic remodelling, especially in fast skeletal muscle, the contractile phenotype assessed by MHC isoform expression is, surprisingly, hardly modified in response to CK deficiency. Both endurance training and voluntary wheel-running activity are known to induce a shift in myosin isoenzyme expression from fast to slower isoforms in rodents, but these changes are moderate compared to the major changes in MHC profile induced by changes in muscle load (Bigard *et al.* 2000; Zoll *et al.* 2003). Here, this was the case in WT mice, where the MHC profile evolved towards an increase in MHC-IIX at the expense of MHC-IIB in mixed plantaris muscle only. It is interesting that $CK^{-/-}$ fast muscles had a higher percentage of MHC-IIX, which was further increased in gastrocnemius of $CK^{-/-}$ active mice. As skeletal muscles of $CK^{-/-}$ mice appear as naturally trained muscles, this cannot explain the decreased voluntary exercise capacity of these animals.

Under conditions of maximal metabolic rate, the locomotor musculature receives 90% of the blood flow and consumes over 90% of the oxygen taken up by the lungs. In turn, mitochondria and capillary blood together determine the aerobic capacity of muscle (Weibel *et al.* 2004). Thus mitochondrial content is an important determinant of endurance capacity. However, there were no obvious signs of increased oxidative capacity in gastrocnemius and soleus muscles of WT active mice compared to sedentary mice. On the other hand, a strong effect of CK deficiency was observed in the gastrocnemius muscle. CK deficiency induces a muscle type-specific profile of adaptation, both at the biochemical and cyto-architectural levels. While soleus muscle mainly exhibits changes in the glycolytic enzyme pattern, fast skeletal muscle shows profound changes in mitochondrial capacity and myoglobin content, with the most prominent effects in $CK^{-/-}$ rather than M-$CK^{-/-}$ mice (van Deursen *et al.* 1993; Veksler *et al.* 1995; Steeghs *et al.* 1997, 1998; de Groof *et al.* 2001*b*,*c*). Eleven-month-old mice showed similar results with an increase in mitochondrial enzymes in gastrocnemius and an increase in H-LDH, the isoenzyme associated with oxidative metabolism, in soleus muscle. A more than three-fold increase in CS, a marker enzyme of mitochondrial content, together with a more than 10-fold increase in cytochrome oxidase, complex IV of the respiratory chain, were observed in gastrocnemius. This muscle compensates for the lack of CK-buffering capacity by (1) increasing mitochondrial content (van Deursen *et al.* 1993; Veksler *et al.* 1995; Steeghs *et al.* 1997; Bruton *et al.* 2003; Kaasik *et al.* 2003), (2) channelling energy supply directly from mitochondria to ATPases involved in the excitation–contraction process (Kaasik *et al.* 2003) and (3) increasing AK activity and activating other phosphotransfer kinases (Dzeja *et al.* 2004). This functional and structural remodelling of mitochondria

plays an active part in rescuing calcium homeostasis and contractile function. However, these adaptive mechanisms appear insufficient to overcome the lack of CK. Indeed, total ATP production rate is still two times lower in $CK^{-/-}$ muscles (Dzeja *et al.* 2004). Moreover, calcium homeostasis, adenine nucleotide handling and contractile function are impaired, showing the limitation of these adaptive mechanisms (Steeghs *et al.* 1997; de Groof *et al.* 2001*a*; Gorselink *et al.* 2001*a*; Kaasik *et al.* 2003).

Additionally, exercise capacity depends on diaphragm muscle function. Indeed, muscle power and work output are profoundly impaired in diaphragm muscle of $CK^{-/-}$ mice (Watchko *et al.* 1997), underscoring the possible implication of respiratory defects in exercise limitation of CK-deficient mice.

CK deficiency and muscle wasting

Muscle damage and decreased muscle mass could also be major determinants of altered voluntary exercise capacity. Decreased force developed per unit cross-sectional area by skeletal muscle of $CK^{-/-}$ mice, due in part to decreased myofibrillar and increased mitochondrial content (Gorselink *et al.* 2001*b*), may participate in muscle weakness. Indeed, a decrease in total MHC of 25% in gastrocnemius and 45% in soleus was also described in $CK^{-/-}$ mice (de Groof *et al.* 2001*b*).

Besides, as noted in previous studies (Gorselink *et al.* 2001*b*), muscle mass of either mixed or oxidative muscles was dramatically decreased in both M-$CK^{-/-}$ and $CK^{-/-}$ mice, despite similar body masses, suggesting an increase in fat mass. A decrease in muscle mass also accompanied the decreased voluntary wheel-running capacity of type IIB MHC-null mutant mice (Harrison *et al.* 2002). Among inbred mouse strains, a significant positive correlation was found between calf mass and running-wheel performance (Lerman *et al.* 2002). Despite an increase in mitochondrial content, the maximal voluntary exercise capacity of CK-deficient mice was lower than that of control mice. In this instance, muscle mass can appear as an additional factor limiting voluntary exercise capacity in CK-deficient mice. Indeed, a correlation between relative soleus or plantaris mass at the end of the exercise period, and the total running distance taken as an index of exercise capacity of all groups of mice, could be observed (Fig. 6). It should be noted that metabolic failure also accounts for decreased running performance given that muscle mass hardly differs between $CK^{-/-}$ and M-$CK^{-/-}$ mice.

Moreover, the structure of CK-deficient mouse skeletal muscles appears to be profoundly altered. The presence of fibres with central nuclei suggests that these muscles are subject to degeneration/regeneration processes. This phenomenon is not linked to a massive change in muscle fibre type as myosin isoforms are barely affected. The presence of fibres with small diameter can be interpreted in two ways. They could reflect an adaptive response in order to decrease intracellular diffusion distances, or be a consequence of fibre degeneration/regeneration processes, or both. The presence of inflammatory cells, infiltrate and collagen deposits further suggest that muscles of $CK^{-/-}$ mice are subject to intense damage, incompletely compensated for by healing/repair processes. These muscle characteristics are reminiscent of the phenotype of many muscular dystrophies. This suggests that the metabolic imbalance induced by CK deficiency causes fibre damage, large areas of collagen deposit, and loss of muscle mass. Defects in energy and calcium homeostasis induced by CK depletion may lead to uncontrolled opening of the permeability transition pores leading to apoptosis (Dolder *et al.* 2003), to the activation of protein degradation through the ATP-dependent ubiquitin-proteasome system and/or the calcium-dependent calpain system, both processes leading to massive skeletal muscle wasting.

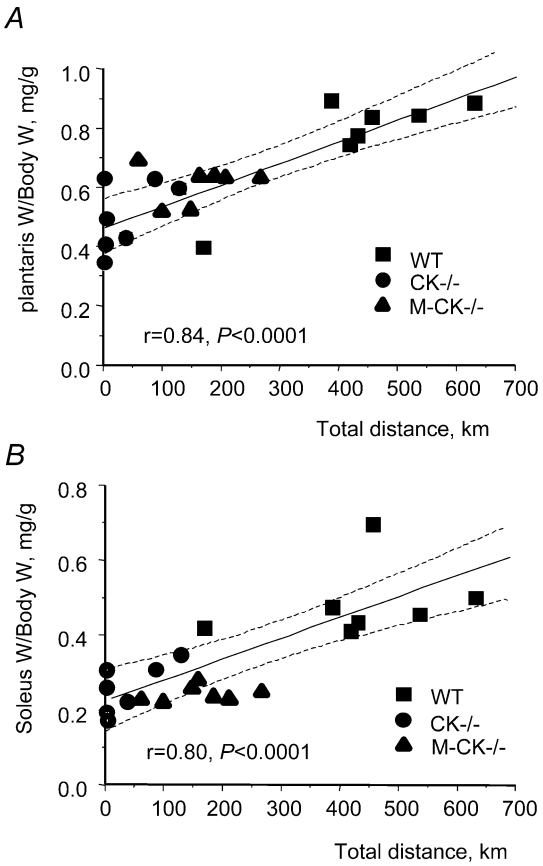

Figure 6. Correlation between muscle mass and exercise capacity
A strong correlation is observed between the between the relative weights of soleus (*A*) or plantaris (*B*) at the end of the exercise period and the total running distance, taken as an index of voluntary exercise capacity of all groups of mice.

Consequences for our knowledge of CK function

It appears that in highly organized and well-differentiated muscle cells, compartmentalizion of adenine nucleotides is of crucial importance. Thus, different evolutionary strategies have been used to overcome ADP-diffusion limitations. One of them is the CK system. The existence of CK and its isoenzymes in all muscles of mammals, the tissue specificity of its expression, its complex organization within cellular architecture, and its fine adaptability depending on muscle function and needs, all underline its critical role in the control of energy fluxes and ion homeostasis in muscle cells, to ensure highly efficient muscle contraction. It seems that morphological and functional adaptations are sufficient to ensure the viability of the $CK^{-/-}$ mice during sedentary laboratory life. However, despite numerous adaptive mechanisms and the presence of additional localized ATP buffering, measurement of voluntary exercise capacity reveals that these systems are not strictly redundant and that CK plays a crucial role.

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