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Presence of (phospho)creatine in developing and adult skeletal muscle of mice without mitochondrial and cytosolic muscle creatine kinase isoforms

H. J. A. in 't Zandt*, A. J. C. de Groof†, W. K. J. Renema*, F. T. J. J. Oerlemans†, D. W. J. Klomp*, B. Wieringa† and A. Heerschap*

Departments of *Radiology and †Cell Biology, University Medical Center Nijmegen, 6500 HB, Nijmegen, The Netherlands

We assessed the relationship between phosphocreatine (PCr) and creatine (Cr) content and creatine kinase (CK) activity in skeletal muscle of mice. The PCr and total Cr (tCr) concentrations, as well as CK activity, in hindlimb muscles of mice, with or without the cytosolic and mitochondrial isoforms of muscle creatine kinase (wild-type or $CK^{--/--}$ mice), were determined by *in vivo* magnetic resonance (MR) spectroscopy and by biochemical means during postnatal growth and adulthood. In wild-type muscle the [tCr], PCr/ATP ratio and CK activity increased rapidly in the first 4–7 weeks. Remarkably, $CK^{--/--}$ mice showed a similar increase in the PCr/ATP ratio during the first month in the presence of only minor brain-type BB-CK activity. Uptake of Cr in muscle was seemingly unrelated to CK activity as tCr increased in the same way in the muscles of both mouse types. At older ages the PCr/ATP ratio decreased in $CK^{--/--}$ muscles, in contrast to wild-type where it still slowly increased, whereas [tCr] was similar for muscle of both mouse types. Using a new *in vivo* MR approach with application of [4-^{13}C]Cr, a lower PCr/tCr ratio was also observed in $CK^{--/--}$ muscle. From these data it follows that *in vivo* global ATP levels at rest are similar in the presence or absence of CK. Although Cr could still be converted to PCr in mature $CK^{--/--}$ muscle, the immediate availability of PCr decreased, and PCr became partly inconvertible at older ages. Apparently, catalysis of the CK reaction by BB-CK, although significant in muscles of newborn mice, gradually declines to very low levels in adulthood. Part or all of this BB-CK may arise from satellite cells fusing with myotubes, a process that is most active during the first months of life. Finally, our observation that the MR and chemical assessment of muscle [tCr] and PCr/tCr ratio were similar for all mice does not support the existence of a significant MR-invisible or immobile pool of Cr, with a role for CK in this phenomenon.

Creatine kinases (CKs; EC 2.7.3.2) form a small family of isoenzymes that catalyse the reaction:

$$\text{Cr} + \text{MgATP}^{2-} \rightleftharpoons \text{PCr}^{2-} + \text{MgADP}^- + \text{H}^+,$$

where Cr is creatine and PCr^{2-} is phosphocreatine. These proteins play a key role in the energetics of excitable tissues, such as muscle and brain, by keeping ATP/ADP ratios balanced and the adenylate pool highly charged (Wallimann *et al.* 1992; Wyss & Kaddurah-Daouk, 2000). Apart from forming a temporal energy buffer, the CK circuit may also act as an intermediate in spatial energy networks (Walsh *et al.* 2001). Other roles of the CK system, which follow from the presence of other CK reactants, are intracellular regulation of pH (Edstrom *et al.* 1982; Wallimann *et al.* 1992) and inorganic phosphate (P_i) levels (Meyer *et al.* 1984).

To study the overall physiological significance of the CK/PCr system in muscle, we have generated mice with null mutations in the muscle-specific cytosolic *M-CK* and mitochondrial *ScCKmit* genes (van Deursen & Wieringa, 1992; van Deursen *et al.* 1993; Steeghs *et al.* 1997). Apart from specific functional losses, some remarkable metabolic adaptations were observed in the muscles of CK-lacking animals (e.g. van Deursen *et al.* 1993; Steeghs *et al.* 1998; de Groof *et al.* 2001). Early in life a significant part of the total skeletal muscle CK activity comes from the BB-CK isoform, and it has been shown for some species that the expression of this isoform becomes gradually suppressed during maturation of skeletal muscle, leaving only trace amounts at adulthood (Eppenberger *et al.* 1983; Trask & Billadello, 1990). Skeletal muscle of adult mice with combined null mutations for *M-CK* and *ScCKmit* ($M-CK^{-/-}/ScCKmit^{-/-}$, further referred to as $CK^{--/--}$)

showed no up-regulation of BB-CK activity. Nevertheless, substantial amounts of PCr were found in these muscles. However, this pool was not accessible for energy buffering (Steeghs *et al.* 1997; Heerschap *et al.* 1999) and moreover, the exchange-flux of phosphate between the PCr and ATP pools measured in MR transfer experiments had dropped to below detection levels in adult animals (Steeghs *et al.* 1998; Heerschap *et al.* 1999).

The main purpose of the present study was to obtain a more precise characterization of the origin and fate of the PCr pool in muscle without muscle-type CKs as compared with PCr in muscle of wild-type mice. It is of particular interest to know what happens with the muscle PCr content in relation to CK activity and total Cr levels. These key parameters in the setting of PCr tissue levels are expected to change in skeletal muscle of mice during postnatal development and aging. To address how they relate to PCr levels in wild-type and $CK^{--/--}$ mice, the total content of Cr (tCr) and PCr in hindlimb muscle was assessed by *in vivo* MR spectroscopy in parallel with biochemical determinations of muscle Cr levels and CK activity as a function of age. To identify whether uptake and phosphorylation of creatine indeed occur in muscle of adult $CK^{--/--}$ mice we introduce a new approach involving the administration of [4-^{13}C]Cr to monitor its phosphorylation *in vivo* by ^{13}C MR spectroscopy. From this approach also the muscle [PCr]/[tCr] ratio can be determined which, together with tCr content and [PCr]/[ATP] ratios, allows for an assessment of *in vivo* ATP levels in a novel way. Finally, we also compared the potential to de-phosphorylate PCr in muscle of wild-type and of double-knockout mice as a function of age. The results provided further clues on the presence of PCr in (developing) skeletal muscle of wild-type mice and of mice without cytosolic MM-CK and mitochondrial ScCKmit. Moreover, the particular results of this study of metabolite levels also have interesting implications for views on Cr mobility and visibility as seen by MR spectroscopy in skeletal muscle.

METHODS

Animals
In this study a comparison was made between mice lacking cytosolic *M-CK* and sarcomeric mitochondrial *ScCKmit* (i.e. $CK^{--/--}$) and wild-type C57Bl/6 controls. All procedures were approved by the Animal Care Committee of the University of Nijmegen and conformed to the Dutch Council for Animal Care. Animals were anaesthetized with 1.2 % isoflurane in a gas mixture of 50 % O_2–50 % N_2O delivered through a facemask. Their body temperature was monitored using a fluoroptic thermometer (Luxtron 712, CA, USA) and was maintained at 36.8 ± 0.5 °C using a warm water circuit.

Magnetic resonance (MR) equipment
The MR experiments were performed on a horizontal 7.0 T magnet (Magnex Scientific, Abingdon, UK), which was equipped with 150 mT m^{-1} shielded gradients, and interfaced to a SMIS (Surrey Medical Imaging Systems, Surrey, UK) spectrometer, operating at 300.22 MHz for ^{1}H, at 121.53 MHz for ^{31}P and at 75.49 MHz for ^{13}C. The receiver channel was slightly modified by using home-built ultra-low noise preamplifiers and a low-loss active transmit/receive switch.

Phosphorus (^{31}P) MR study of postnatal development
For non-localized ^{31}P MR measurements, a three-turn solenoid coil with a diameter of 8 mm was used with an Alderman-Grant type ^{1}H coil surrounding it (Alderman & Grant, 1979). For young mice (7–14 days of age), a special plastic insert was used to ensure that only the hindlimb muscles were measured. After the shimming procedure, a scout image was obtained in three perpendicular directions to ensure that only the hindlimb was situated in the radio-frequency (RF) field of the coil. Experiments on a phantom (100 mM inorganic phosphate solution) showed that the field of view of the ^{1}H coil corresponded to that of the ^{31}P coil. This was verified by the combined application of localized ^{31}P MR spectroscopy (Ordidge *et al.* 1986) and ^{1}H MRI, which showed the absence of ^{31}P MR signals outside the phantom. MR spectra were acquired using a 90 deg RF pulse of 40 μs with a repetition time of 7 s and were obtained from the hindlimb of mice starting at ages 7 days up to 300 days ($n = 3$–6 for each data point). Additional spectra were obtained with a repetition time of 25 s from animals at ages 90 and 110 days to obtain metabolite levels under fully relaxed conditions and to assess saturation factors ($n = 3$ for each mouse type).

Post-mortem study
^{31}P MR spectra were acquired from wild-type mice aged 21 days and 12 months ($n = 2$ each), and $CK^{--/--}$ mice aged 21 days ($n = 3$) and 6, 9 and 12 months ($n = 1$ per time point). The ^{31}P MR spectra ($T_r = 7$ s, 128 scans) were acquired using the same coil setup as used in the postnatal development study. After the acquisition of a reference spectrum, the animal was killed with an overdose of isoflurane and the PCr resonance was monitored until its level became constant.

In vivo monitoring of [4-^{13}C] creatine by ^{13}C magnetic resonance spectroscopy (MRS)
$CK^{--/--}$ ($n = 5$) and wild-type ($n = 5$) mice between 3 and 6 months of age were injected intravenously with 100 % [4-^{13}C]Cr (11.25 mg ml^{-1}, 200 μl), three times a week, for 3 weeks. The labelled Cr was obtained from Mercachem (Mercachem, Nijmegen, the Netherlands) and synthesized according to Han & Sillerud (1986). Hindlimb muscles of these mice were analysed weekly by ^{13}C MR spectroscopy, using a 4-turn solenoid ^{13}C coil with a diameter of 8 mm surrounded by an Alderman-Grant ^{1}H coil. ^{13}C MR spectra were acquired with a 90 deg RF pulse of 40 μs, a repetition time of 3 s, and Waltz-4 ^{1}H decoupling (40 W, duty cycle 0.7 %) to ensure that the resonances of PCr and Cr are maximally resolved. Heating caused by decoupling power was evaluated by monitoring the temperature of the hindlimb and of the body by a two-channel fluoroptic thermometer (Luxtron 712); no warming of the animal could be detected during 1.5 h.

Ischaemia protocol
To make hindlimb muscles ischaemic the MR probe was provided with a diaphragm plate, which allows reversible and reproducible occlusion of the hindlimb (in 't Zandt *et al.* 1999). Before, during and after ischaemia, sequential ^{13}C MR measurements were made essentially as described above, except that the number of scans was reduced to 600 with a repetition time of 1 s. These experiments were performed on mice between 3 and 6 months of age.

Quantification of the total Cr pool in muscle using localized ^{1}H MR spectroscopy

Creatine levels were determined by quantitative localized ^{1}H MRS in gastrocnemius muscle, oriented at 55 deg with respect to the static magnetic field (B_o) direction (magic angle) to avoid complications due to dipolar coupling (in 't Zandt *et al.* 2000*b*). Localized ^{1}H MRS was performed as described previously (in 't Zandt *et al.* 2000*b*). A stimulated echo pulse sequence with an echo time of 10 ms and a repetition time of 5 s was used to select a voxel (typical size $2 \times 2 \times 2$ mm^3) in the gastrocnemius muscle, guided by MR images acquired in three oblique, perpendicular directions. Localized shimming on this (oblique) voxel was performed until an H$_2$O peak with full width at half maximum of less than 22 Hz was reached. Creatine levels were estimated from the peak area of its methyl protons using the ^{1}H signal of water as an internal reference and assuming a muscle water content of 76 % (Sjogaard & Saltin, 1982). Corrections were made for T_2 transverse relaxation times. The relaxation time T_2 for Cr methyl and water proton spins was estimated by the acquisition of ^{1}H MR spectra at different echo times and applying a mono-exponential fit to the signal intensities obtained at increasing echo times. At a repetition time of 5 s, the proton spin system was considered to be fully relaxed and hence no correction for T_1 was applied. Creatine content was determined in this way at the ages of 60, 120 and 200 days ($n = 3$ each).

Biochemical determination of CK activity and metabolite levels

Mice were killed by cervical dislocation and hindlimb muscles snapshot frozen and stored at -80 °C. Muscles were homogenized in a Teflon–glass Potter-Elvehjem using 10 volumes of an ice-cold buffer containing 200 mM sucrose, 2 mM EDTA, 10 mM TrisHCl (pH 7.4), supplemented with heparin (50 units ml^{-1}) and protease inhibitors (Boehringer Mannheim, Germany). The suspension was diluted 1:1 in a 30 mM phosphate buffer (pH 7.4), with 0.2 mM DTT, 0.05 % (v/v) Triton X-100 and protease inhibitors. Extracts were incubated at room temperature for 20 min and subsequently centrifuged at 15 000 *g* (Eppendorf) at 4 °C for 20 min. Total CK activity was measured at 37 °C using a CK-NAC-activated kit (Boehringer) and expressed in mmol (l intracellular water)$^{-1}$ s^{-1}, assuming 20 % protein content in skeletal muscle (Zuurveld *et al.* 1985) and 1 g of muscle being equivalent to 0.69 ml intracellular water. For zymogram analysis, total protein extracts (5 μg) were resolved on a 0.7 % (v/v) Seakem ME agarose gel (FMC Bioproducts, Rockland, ME, USA). Enzyme activities were visualized by a CK isoenzyme colourimetric detection kit (Sigma Diagnostics, St Louis, USA (Steeghs *et al.* 1997)).

Figure 1. ^{31}P MRS of hindlimb skeletal muscle as a function of age

A, spectral changes for a wild-type (left panel) and a *CK*$^{--/--}$ mouse (right panel) reflect postnatal development of high energy phosphate metabolism. Spectra are vertically scaled to the ATP content. The age of the animals (days) is indicated at the left. PME, phosphomonoesters; P$_i$, inorganic phosphate; PDE, phosphodiesters; PCr, phosphocreatine; ATP, adenosine triphosphate. *B*, the PCr to ATP signal ratio as a function of age for *CK*$^{--/--}$ (○) and control (■) mice. Ratios are not corrected for partial signal saturation. Each data point represents the average of at least three different mice. Standard deviations are indicated when outside the symbol sizes.
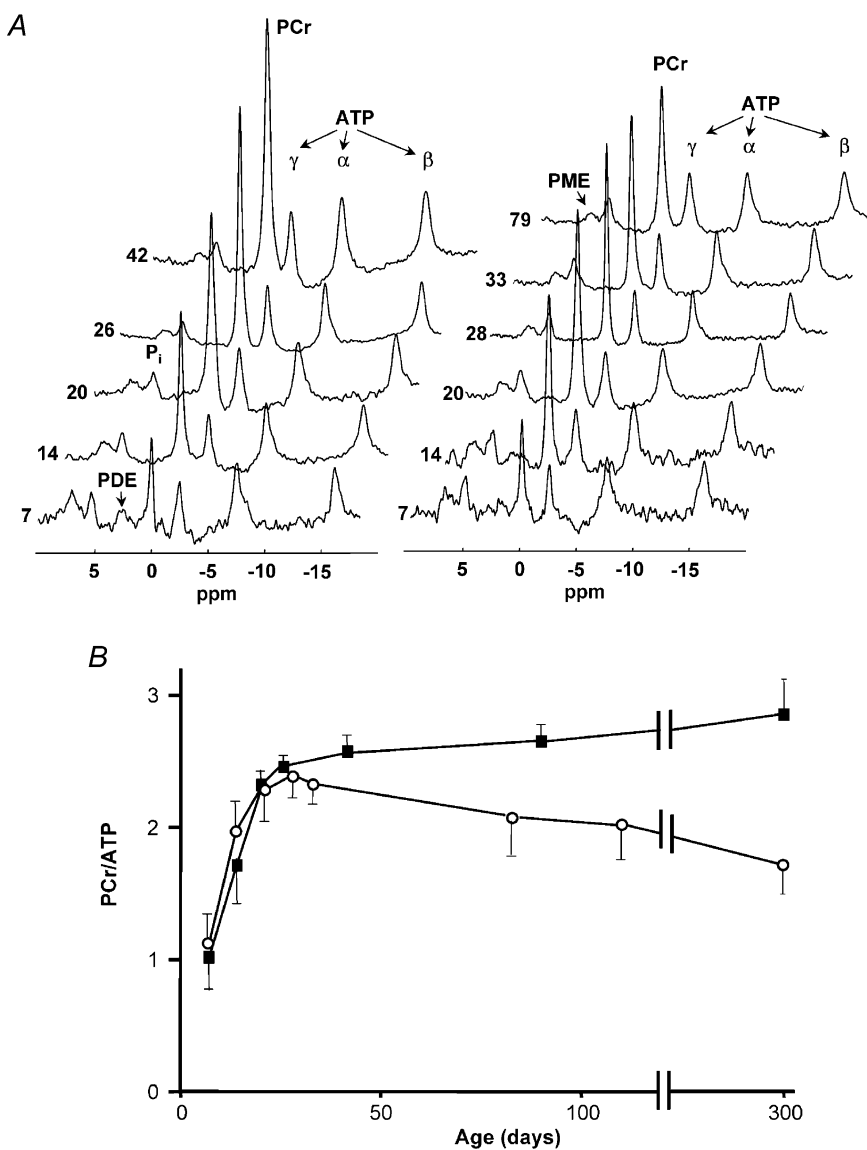
For chemical analysis of the Cr content in the gastrocnemius–plantaris–soleus (GPS) complex, tissues were extracted by 0.6 M $HClO_4$ and neutralized with 3 M KOH. Total Cr levels were assayed via coupled enzymatic reactions using a spectrophotometer operating at 340 nm (Bergmeyer, 1974). Creatine levels were determined in μmol (g wet weight)$^{-1}$ and mmol (l tissue)$^{-1}$, assuming a muscle density of 1.06 g ml^{-1} (Mendez & Keys, 1960). These were converted to mmol l^{-1} intracellular water assuming a cellular water fraction of 0.73 (Sjogaard & Saltin, 1982; Forsberg *et al.* 1991). PCr was determined as described previously (Steeghs *et al.* 1997; Steeghs *et al.* 1998).

MR data analysis

MRS data were evaluated in the time domain using MR user interface (MRUI) 97.1 software:

http://www.mrui.uab.es/mrui/mruiHomePage.html

The processing of ^{1}H MR spectra was performed as described previously (in 't Zandt *et al.* 2000*b*). The creatine peak at a chemical shift of 3.03 parts per million (p.p.m.) was fitted assuming a Gaussian line shape model function. Resonances in ^{31}P MR spectra were also fitted with a Gaussian line shape model function. No further prior knowledge was used. For the assessment of ATP content only the γ-ATP and the β-ATP signals were considered. Intracellular pH was derived from the chemical shift difference between P_i and PCr peaks (Heerschap *et al.* 1988)

In ^{13}C MR spectra, all signals originating from carbons in lipids were filtered using Hankel Lanczos singular value decomposition

(de Beer & van Ormondt, 1992), leaving Cr and PCr signals around 157 p.p.m. for further analysis. As prior knowledge, a chemical shift difference of 0.8 p.p.m. (Han & Sillerud, 1986) and equal damping for these resonances was assumed.

Differences between mean parameter values were analysed by Student's unpaired *t* test.

RESULTS

Postnatal development of (phospho)creatine levels

Energy metabolites in developing hindlimb muscles of control and $CK^{--/--}$ mice aged 7–300 days were monitored by ^{31}P MR spectroscopy. All ^{31}P MR spectra (Fig. 1*A*) showed signals for PCr, γ-, α- and β-ATP, inorganic phosphate (P_i) and phosphomonoesters (PME). During the first 4 weeks of life the PCr/ATP signal ratio gradually increased to a value of about 2.5 (uncorrected for spin saturation), with a similar slope for wild-types and mutants (Fig. 1*B*). Thereafter, this ratio slightly increased further in controls, but gradually declined in $CK^{--/--}$ mice.

To assess how the size of the cellular Cr pool determines the ratio of PCr over ATP we measured total Cr levels as a function of age, in the same cohort of mice as studied by ^{31}P MRS. Using a chemical assay the creatine content appeared to increase similarly in both wild-type and

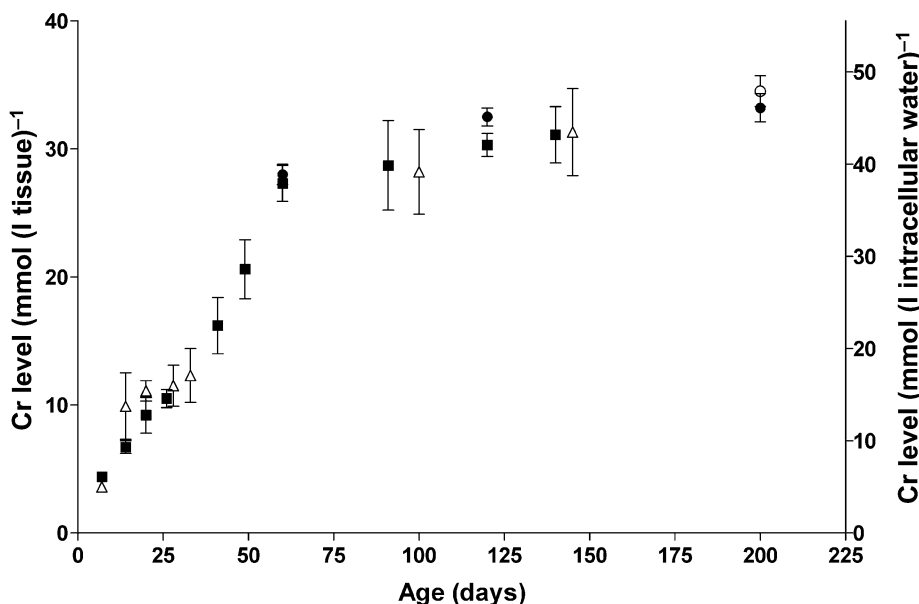**Figure 2. Creatine levels in hindlimb skeletal muscle as a function of mouse age**

Data are presented as mmol (l tissue)$^{-1}$ (left axis) and as mmol (l intracellular water)$^{-1}$ (right axis) assuming an intracellular water fraction of 0.73 (see Methods). Values for $CK^{--/--}$ mice are indicated by open symbols and for wild-type control mice by filled symbols. Data points at 60, 120 and 200 days, indicated by filled circles, were obtained by quantitative ^{1}H MRS of wild-type gastrocnemius muscle (*n* = 3) and at 200 days of age also in $CK^{--/--}$ muscle (*n* = 3), indicated by an open circle. The other data points have been obtained by biochemical means from the muscle GPS complex. The data points at 60 and 120 days (*n* = 3) have been determined from the same leg on which the MRS quantification was performed. The data points at 140 and 145 days are averages of six and five mice, respectively, varying in age between 120 and 160 days. Each other point represents an average of 3–6 mice, except at 7 days postpartum, which are single measurements. Standard deviations are indicated if extending beyond the symbol size.

$CK^{-/--}$ mice during postnatal development and levelled off after about 7 weeks of age at values between 27 and 35 mmol (l tissue)$^{-1}$ (see Fig. 2). Total creatine was also determined by quantitative localized ^{1}H MRS, with the muscle oriented at the so-called magic angle (Kreis *et al.* 1997; in 't Zandt *et al.* 2000*b*), from the peak area of its methyl protons and using the water peak as a reference. The T_2 relaxation times used for signal correction were estimated to be 83 ± 4 ms ($n = 6$) for creatine methyl protons and 21.9 ± 0.1 ms ($n = 6$) for water proton spins. At 60 and 120 days of age, tCr levels were determined in this way by MRS and also chemically, in the same skeletal muscles ($n = 3$). At 60 days of age, total creatine concentration was 27.9 ± 0.9 mmol (l tissue)$^{-1}$ by MRS, which is not different from the creatine tissue content determined chemically $(27.3 \pm 1.4$ mmol (l tissue)$^{-1})$. Although the average value determined by MRS at 120 days $(32.5 \pm 0.7$ mmol (l tissue)$^{-1})$ is somewhat higher than that determined chemically (30.3 $\pm$ 0.9 mmol

(l tissue)$^{-1}$) it is within the range of chemically determined values between 90 and 160 days of age (see Fig. 2). ^{1}H MRS analysis at 200 days of age found tCr to be 33.2 ± 1.1 mmol (l tissue)$^{-1}$ in controls ($n = 3$) and 34.5 ± 1.2 mmol (l tissue)$^{-1}$ in $CK^{-/--}$ muscles ($n = 3$).

Postnatal changes in creatine kinase activity

During postnatal development of mammalian skeletal muscles a gradual repression of *B-CK* gene expression takes place. In order to assess the possible involvement of this CK family member in PCr pool build-up and depletion, we quantified the residual level of BB-CK isoenzyme in $CK^{-/--}$ muscles in relation to normal wild-type CK activity over time. The zymogram assay confirmed complete absence of MM-CK and ScCKmit in double mutant muscle, whereas the BB-CK isoenzyme was clearly visible in both wild-type and mutant muscle extracts at 7 and 14 days of age (Fig. 3*A*). During further progression to adulthood, isoenzyme staining on zymograms dropped to undetectably low levels, but the
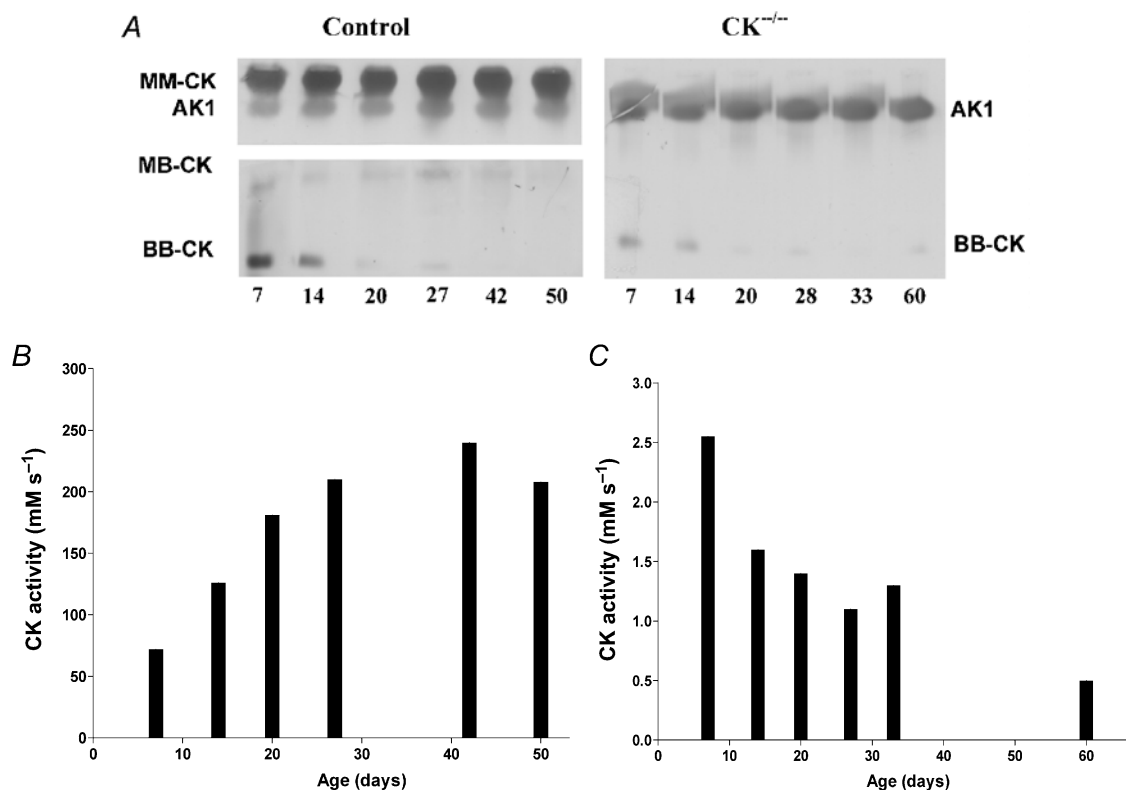

Figure 3. CK isoenzyme and adenylate kinase 1 (AK1) distribution in skeletal muscle as a function of mouse age

A, zymogram analysis of extracts from wild-type (left panel) and $CK^{-/--}$ mouse muscle (right panel) reveals low levels of BB-CK activity during the first 2 weeks. Note the complete absence of MM-CK activity in $CK^{-/--}$animals. AK1 activity staining appears different in the right and left panels due to the use of differential staining protocols, necessary to avoid overexposure of MM-CK signal and blurring of the AK1 band by the presence of vast amounts of MM-CK in wild-type muscles. Staining of (low) ScCKmit activity is not visible under the present conditions. *B*, CK enzyme activity measured as a function of age in wild-type muscle. Activities are given as mmol (l intracellular water)$^{-1}$ s^{-1}. *C*, CK activity in $CK^{-/--}$ muscle, which is the non-MM-CK/ScCKmit activity, expressed as mmol (l intracellular water)$^{-1}$ s^{-1}. Note the different scaling from *B*.

corresponding residual BB-CK enzyme activity could still be assessed with spectrophotometric analysis of tissue lysates. In wild-type hindlimb skeletal muscle, CK activity increases during the first month of life from about 75 mM s^{-1} at an age of 7 days to a value of about 200 mM s^{-1} at adulthood (Fig. 3*B*). In 7-day-old $CK^{--/--}$ muscles this activity was about 2.5 mM s^{-1} or 4 % of the normal overall CK activity (combined BB-CK, MM-CK and ScCKmit activities in wild-type controls) and decreased to values of less than 1 mM s^{-1} within about 40 days (Fig. 3*C*).

Uptake and phosphorylation of ^{13}C-labelled creatine in muscle of adult mice

To assess the uptake and phosphorylation of creatine in mature wild-type and $CK^{--/--}$ mice we explored a new approach by which we could assess Cr and PCr simultaneously in muscle *in vivo* using ^{13}C MR spectroscopy after injection of [^{13}C]Cr. After 1 week, this resulted in well-resolved signals in the ^{13}C MR spectrum of wild-type muscle for Cr and PCr (Fig. 4*A*). Strikingly, uptake and phosphorylation of [^{13}C]Cr were also detectable in $CK^{--/--}$ mice (Fig. 4*B*). The phosphorylated fraction of the Cr pool remained constant during 3 weeks of creatine injection in both mouse strains (Fig. 4*C*). The average PCr/tCr ratio as determined by ^{13}C MRS in $CK^{--/--}$ mice (0.56 ± 0.05) was significantly lower than in wild-

type muscle (0.74 ± 0.04). In a determination by chemical analysis (Steeghs *et al.* 1997) similar values were found for $CK^{--/--}$ mice (0.55 ± 0.06) and slightly lower values for wild-types (0.66 ± 0.07), which is probably due to some PCr breakdown during the extraction procedure because of the high CK activity.

To verify whether the [^{13}C]PCr in muscle is metabolically active, we applied ischaemia to the hindlimb muscle. ^{13}C MR spectra were acquired at a time resolution of 10 min, during a total ischaemic period of 20 min. In wild-type mice we observed a clear conversion of PCr to Cr during this time interval, and full recovery of PCr levels occurred after reperfusion (Fig. 4*D*). In $CK^{--/--}$ mice, no changes in PCr and Cr levels could be observed, within limits set by the signal-to-noise ratio.

Non-invasive estimation of intracellular ATP and ADP content at rest

Usually the *in vivo* levels of compounds in skeletal muscle involved in the CK reaction are assessed by ^{31}P MRS by taking the chemically determined contents of ATP and total creatine as starting points. The availability in this study of absolute Cr levels and of the [PCr]/[tCr] and [PCr]/[ATP] ratios allowed the non-invasive estimation of PCr and ATP levels. The average [tCr] for muscle of wild-type mice between 60 and 200 days of age was
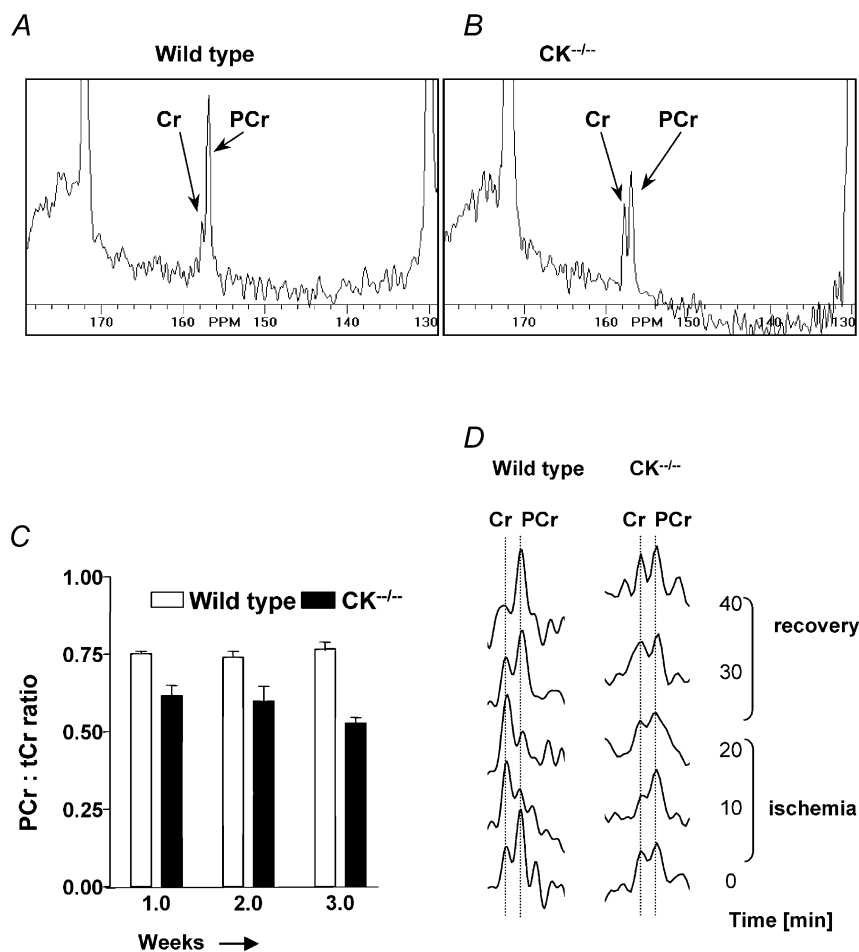

Figure 4. ^{13}C MRS of adult hindlimb muscle, after administration of [4-^{13}C]Cr

^{13}C MR spectra (128–180 p.p.m. spectral range) of wild-type control (*A*) and $CK^{--/--}$ mice (*B*). *C*, PCr/tCr ratios derived from these spectra assessed over a 3 week period. *D*, zoomed ^{13}C MR spectra obtained before and during 20 min of ischaemia and recovery. Hydrolysis of PCr and rephosphorylation of Cr are evident in wild-type muscles, while in $CK^{--/--}$ muscles no conversion was detected within the limits set by the signal-to-noise ratio.

Table 1. Average metabolite concentrations, ratios and tissue pH for adult mouse skeletal muscle

	Wild type		M-CK/ScCKmit knock-out ($CK^{-/--}$)	
	mmol (l tissue)$^{-1}$	mmol (l intracellular water)$^{-1}$	mmol (l tissue)$^{-1}$	mmol (l intracellular water)$^{-1}$
[tCr][a]	30.5 ± 2.0	41.8 ± 2.7	31.4 ± 2.8	43.0 ± 3.8
PCr/tCr[b]*		0.74 ± 0.04		0.56 ± 0.05
[PCr]	22.5 ± 1.9	30.8 ± 2.6	17.6 ± 2.2	24.1 ± 3.0
PCr/ATP[c]*		2.90 ± 0.19		2.36 ± 0.21
[ATP]$_{MRS}$	7.8 ± 0.8	10.7 ± 1.1	7.5 ± 1.1	10.3 ± 1.5
[ATP]$_{chem.}$[d]*	8.2 ± 0.8	11.2 ± 1.1	5.7 ± 0.6	7.8 ± 0.8
pH[e]		7.22 ± 0.03		7.24 ± 0.04
[ADP][f]		0.037		(0.083)

All the data (± S.D.) are obtained from measurements of animals between 60 and 200 days of age. Metabolite concentrations in mmol (l tissue)$^{-1}$ (left column for each mouse type) were converted to mmol (l intracellular water)$^{-1}$ (right columns) assuming a fraction of 0.73 for intracellular water. [PCr] and [ATP]$_{MRS}$ were derived from the PCr/tCr and PCr/ATP ratios, respectively. *Difference comparison between wild-type and $CK^{-/--}$ by unpaired t test: two-tailed P value < 0.0001. [a]tCr: phosphorylated + unphosphorylated creatine. [b]Data from chemical and ^{1}H MRS determinations have been taken together. $n = 24$ for wild type and 12 for $CK^{-/--}$. Data from ^{13}C MRS of the hindlimb, averaged from measurements obtained 1, 2 and 3 weeks after the start of [4-^{13}C]creatine administration for four wild type and four $CK^{-/--}$ mice. $n = 11$ for each mouse species. [c]Ratios obtained from ^{31}P MR spectra measured under relaxed spin conditions ($T_r > 20$ s) or corrected for spin saturation if obtained at a pulse repetition time of 7 s. $n = 9$ for wild type and $CK^{-/--}$. [d]Data taken from Steeghs (1995) in μmol (g wet weight)$^{-1}$ and converted to mmol (l intracellular water)$^{-1}$ assuming a muscle density of 1.06 and an intracellular water fraction of 0.73. $n = 5$ for wild type and $n = 6$ for $CK^{-/--}$. [e]Determined from ^{31}P MRS data. $n = 6$ for wild type and $n = 5$ for $CK^{-/--}$. [f]ADP is estimated as described in the text, using the [ATP]$_{MRS}$ values and assuming the CK reaction to be at equilibrium (this may not be valid for $CK^{-/--}$).

30.5 ± 2.0 mmol (l tissue)$^{-1}$ (see Table 1). With a PCr/tCr ratio of 0.74 ± 0.04 we calculate an average value for [PCr] of 22.5 ± 1.9 mmol (l tissue)$^{-1}$ and with a PCr/ATP ratio of 2.90 ± 0.19 we arrive at an average [ATP] of 7.8 ± 0.8 mmol (l tissue)$^{-1}$. In previous studies we obtained similar [ATP] values for wild-type skeletal muscle (GPS complex) by chemical means: i.e. 8.2 ± 0.8 mmol (l tissue)$^{-1}$ (Steeghs, 1995) and 8.3 ± 0.5 mmol (l tissue)$^{-1}$ (van Deursen *et al.* 1993). [ATP] in $CK^{-/--}$ muscle derived by the MRS approach was not different from wild-types, but it was substantially lower in the chemical determination. Assuming a fraction of 73 % for intracellular water the intracellular concentrations of tCr, PCr and ATP were also determined (Table 1). Free intracellular ADP levels were derived from these concentrations and the CK reaction assuming this reaction to be at near-equilibrium with an equilibrium constant of 1.66×10^9 (Veech *et al.* 1979) and including intracellular pH data from ^{31}P MR spectra of skeletal muscle. The near-equilibrium condition generally seems valid for skeletal muscle at rest (e.g. Veech *et al.* 1979). This condition also appears to occur in muscle of wild-type mice (van Deursen *et al.* 1993), but may not necessarily be valid for $CK^{-/--}$ muscle.

Post-mortem de-phosphorylation of phosphocreatine as a function of age

Having demonstrated that Cr phosphorylation can occur in both developing and mature muscle of $CK^{-/--}$ mice at

Figure 5. Post-mortem depletion of PCr in mouse skeletal muscle followed by ^{31}P MRS

The animals were killed at $t = 0$ as described in the text. Data point symbols for wild-type are: ■, 21 days ($n = 2$); ●, 12 months ($n = 3$). Those for $CK^{-/--}$ are: □, 21 days ($n = 3$); ▽, 3 months ($n = 1$); △, 9 months ($n = 2$); ○, 12 months ($n = 1$). In control mice at 21 days and 12 months of age the decay is the same. In $CK^{-/--}$ muscle, the ability to hydrolyse PCr decreases with age. In 21-day-old $CK^{-/--}$ mice, PCr gets completely depleted, but at 3 months or older, PCr appears to decrease only to 50 % and at a lower rate.
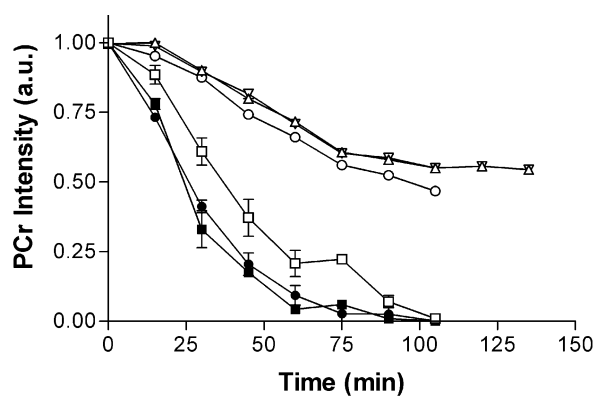
rest, we next investigated to what extent also the inverse reaction, i.e. de-phosphorylation of the PCr pool, could still ensue in these animals. We therefore quantified this activity as a function of age by measuring the rate of PCr signal decay in hindlimbs after a maximal physiological challenge: the onset of death in the MR magnet. Post-mortem PCr breakdown occurs at the same rate in 21-day- and 12-month-old wild-type mice (Fig. 5). In contrast, $CK^{--/--}$ mice showed delayed depletion of PCr at the age of 21 days, while de-phosphorylation of PCr was further slowed down at 3, 9 and 12 months of age (Fig. 5). In these latter measurements, the PCr signal did not drop to zero, but reached a plateau at about 50 % of the initial concentration, indicating that on the time scale of the experiment a significant portion of PCr had become metabolically inactive.

DISCUSSION

Differences in PCr, total Cr content and CK activity during development and at adulthood

The increase of the CK activity and of the PCr/ATP ratio during postnatal development of skeletal muscle in wild-type mice confirms results of previous studies in rodents (e.g. Zuurveld *et al.* 1985; Heerschap *et al.* 1988). In the present study we also observed a postnatal increase for total muscle creatine (tCr) content in these mice, which in the first 4 weeks occurs in parallel with increasing CK activity and PCr/ATP ratios. In general for different tissues there appears to be a positive correlation between CK activity and Cr content (Wyss & Kaddurah-Daouk, 2000). However, it is clear that this correlation does not hold strictly. An increasing tCr level paralleled a minor and steadily decreasing CK activity in the $CK^{--/--}$ mice, which declined to levels below 1 mM s^{-1}. A decline of BB-CK activity has also been observed for the hearts of these mice (Saupe *et al.* 2000). Interestingly, the PCr/ATP ratio in skeletal muscle of $CK^{--/--}$ mice increased in a very similar fashion to wild-type mice during the first 4 weeks after birth, despite the fact that the total CK activity differed profoundly between mutants and controls. It appears that the initial increase in PCr levels relates to the postnatal increase in the tCr pool, which is largely identical in wild-type and $CK^{--/--}$ mice. While beyond 30 days of age the PCr/ATP ratio increased more slowly in wild-type or even decreased in $CK^{--/--}$ mice, the increase of the muscle tCr content levelled off somewhat later. Resting PCr/ATP ratios are not only determined by the tCr level and CK activity (see below), but also by the intracellular energy charge and total adenylate levels, which may change during the postnatal development of skeletal muscle (e.g. Isselhard *et al.* 1973). The present study indicates that Cr uptake in skeletal muscle is independent of its phosphorylation by CK. This uptake is an active process, regulated by specific Na$^+$–Cr co-transporters (Guerrero-Ontiveros & Wallimann, 1998; Wyss & Kaddurah-Daouk,

2000), the activity of which is coupled to energy- and Na$^+$-, K$^+$- and Ca^{2+}-homeostasis. Creatine transporter activity shows regional and developmental differences (Kekelidze *et al.* 1999; Murphy *et al.* 2001), but it seems unaffected in CK-lacking mutants.

Once $CK^{--/--}$ mice become older than 4 weeks, they display lower PCr/ATP and PCr/tCr ratios than wild-type mice. To put this in the proper perspective we first consider how CK activity, the *in vivo* flux through the CK reaction and ATPase activity relate to each other. The maximum activity (V_{max}) of CK from adult wild-type GPS muscle is about 200 mM s^{-1} at 37 °C (see Fig. 3*B*). This is in agreement with other studies on rodent (fast twitch) skeletal muscle (e.g. Zuurveld *et al.* 1985; Bittl *et al.* 1987; van Dorsten *et al.* 1996), but it is in contrast to the actual resting CK flux *in vivo* as derived from ^{31}P MRS magnetization transfer in our earlier studies (van Deursen *et al.* 1993), which is approximately 8 mM s^{-1} and thus only about 5 % of the *in vitro* values. Similar discrepancies have been noted previously for skeletal muscle and are explained by specific kinetic behaviour of CK enzymes under *in vivo* conditions (Bittl *et al.* 1987; McFarland *et al.* 1994; Nicolay *et al.* 1998). Still, these fluxes are well above maximum values of ATPase activity so that adequate backup during the early phase of muscle contraction is ensured. In the cytosolic *M-CK* single-knockout mice the *in vitro* CK activity at 37 °C drops to about 5 mM s^{-1} and the *in vivo* CK flux to below 0.4 mM s^{-1} (van Deursen *et al.* 1993, 1994; van Dorsten *et al.* 1996; Saupe *et al.* 1998). These mice lack muscle burst activity as demonstrated by electrical stimulation at 5 Hz. As the rate of ATP breakdown may easily increase above 1 mM s^{-1} at such muscle activity (calculated from data in Hood *et al.* (1986) and Foley & Meyer (1993)), the CK flux cannot follow ATP breakdown during the first 10 s of contraction when ATP regeneration nearly completely relies on PCr (Foley & Meyer, 1993). The concomitant ADP increase may explain why burst activity is lacking in the muscle of these mice (Roman *et al.* 2002). In muscle of adult $CK^{--/--}$ mice the *in vitro* CK activity is further reduced to less than 1 mM s^{-1}. These mice not only lack burst activity but in addition showed lower muscle force and slower relaxation compared with the *M-CK* single-knockout mice (Steeghs *et al.* 1997). If the *in vivo* activity is also only 5 % of the *in vitro* value (i.e. ~0.02 mM s^{-1}) this comes close to ATPase flux rates as estimated for resting rat skeletal muscle, which are between about 0.01 and 0.04 mM s^{-1} (Meyer *et al.* 1985; Hood *et al.* 1986; Bittl *et al.* 1987; McFarland *et al.* 1994). With similar ATPase fluxes occurring in resting $CK^{--/--}$ muscle the CK reaction might be out of equilibrium upon work transitions, which, at comparable total Cr and adenylate pools, would result in an altered PCr/ATP ratio. In addition this altered ratio could represent a situation in which the (entire) muscle pool of CK substrates no longer is in continuous open contact with the, now only sparsely

present, CK molecules, for instance due to compartmentalization such as discussed for the presumed role of satellite cells below. If, on the other hand, the residual CK activity would still be sufficient to keep the overall CK reaction at or near equilibrium in the resting state, it follows that the concentration of ADP must differ between wild-type and mutant muscle as tissue pH, total creatine and ATP levels are similar. Under equilibrium conditions we estimate an increase of resting free ADP levels by a factor of about 2 in $CK^{--/--}$ muscle (see Table 1). As resting PCr content may vary with fibre type (Kushmerick *et al.* 1992), the different PCr levels might be related to an overall change in fibre type composition in the mutant mouse compared with control. Moreover, postnatal changes in CK activity appear to correlate with changes in myosin heavy chain phenotype (Watchko *et al.* 1996). However, the myosin heavy chain distribution did not appear to differ much in skeletal muscle of $CK^{--/--}$ mice, although a shift towards a more oxidative character of mainly the type II fast twitch glycolytic muscles in $CK^{--/--}$ mice has been identified (Steeghs *et al.* 1998; de Groof *et al.* 2001).

On the origin of the Cr (de)phosphorylation activity in mature muscle of $CK^{--/--}$ mice

As the ^{13}C MRS experiments showed that even in mature $CK^{--/--}$ muscle older than 3 months Cr can be converted to PCr, the question remains of what the origin of this catalytic activity is. It is conceivable that BB-CK activity arises from non-muscle cells, such as vascular endothelium, or that some unknown Cr phosphorylating activity is present in muscle cells. These possibilities seem rather unlikely, in particular as mice with combined deficiency of cytosolic BB-CK and mitochondrial ubiquitous-CK (UbCKmit) isoforms completely lack PCr in the brain (in 't Zandt *et al.* 2000*a*). This provides indirect evidence for the supposition that Cr $\rightleftharpoons$ PCr conversion in mammalian tissues becomes fully blocked if activity of all CK isoforms is completely ablated.

If we assume that Na^+–Cr co-transporters do not transport PCr, formed elsewhere and transported by circulation, into muscle directly, and that the negatively charged PCr (PCr^{2-}) will not freely pass the sarcolemma against a concentration gradient, we can only explain our results by assuming that trace CK activity in adult CK double-mutant muscles causes PCr to occur. Two possibilities remain for this residual CK activity. The first is that even in mature myotubes the expression of the *B-CK* gene, although decreasing with age, is still not completely blocked. Another interesting explanation for the build-up of a PCr pool in mature hindlimb muscle would be the deposition of BB-CK molecules from satellite cells in the process of fusion (Schultz & McCormick, 1994). Muscle satellite cells are prominently present and active in the growing muscles of young animals, but their population decreases with age (Schultz & McCormick, 1994). Satellite cell nuclei are 30 % of the total muscle nuclei at birth, 4 % at 8 months and 2.4 % at 30 months of age in mouse soleus muscle. Moreover, at adult age when muscle growth has slowed down the remaining satellite cells become quiescent, only being re-activated when myofibres are over-challenged. Thus in the adult stage when little satellite fusion is occurring, a considerable fraction of myofibres may hardly have any BB-CK left. This view is corroborated by the post-mortem data of $CK^{--/--}$ muscle, which shows that at older age the rate of PCr de-phosphorylation is slower and that 50 % of the total PCr pool size virtually is unaffected during the observation period. If a significant part of the total cellular Cr pool has no access to the enzyme, this would indeed explain the lower PCr/tCr ratio in adult mutants. In this context $CK^{--/--}$ mice could become a convenient model to study the role of satellite cells, using BB-CK and PCr as sensitive reporters for their fate under different physiological conditions.

Estimation of *in vivo* ATP content using 4-^{13}C-PCr/tCr ratios

From the experiments with [^{13}C]creatine the *in vivo* PCr/tCr ratio was derived for skeletal muscle. Because we also have total Cr levels and PCr/ATP ratios available this gives the opportunity to estimate *in vivo* ATP levels in a novel manner. The tissue concentration derived in this way (~8 mmol (l tissue)$^{-1}$) is in the order of ATP values for (fast twitch) skeletal muscle that have been reported by others (e.g. Shoubridge *et al.* 1984; Meyer *et al.* 1985; Kushmerick *et al.* 1992) and conforms with ATP tissue concentrations determined by chemical means in wild-type mouse skeletal muscle (van Deursen *et al.* 1993; Steeghs, 1995). Moreover, in this way we demonstrate that ATP levels in wild-type and $CK^{--/--}$ muscle are similar, and that differences seen previously in chemical analysis (Steeghs, 1995; Steeghs *et al.* 1997) can be explained by rapid breakdown of ATP during the clamping procedure for the extraction (Meyer *et al.* 1985), which cannot be compensated in $CK^{--/--}$ muscle, because it has a very inefficient CK reaction. In contrast in wild-type muscle PCr is more depleted during this procedure, thus sustaining ATP levels, which is reflected in a slightly lower PCr/ATP ratio when determined by chemical means (Steeghs *et al.* 1997).

The MR visibility of creatine

The present results also bear on the issue of MR visibility of the cellular Cr pool, and the presumed existence of significant free-Cr pools that do not participate in the CK reaction. Based on experiments using ^{14}C-labelled Cr in heart muscle (Lee & Visscher, 1961; Savabi, 1988) and in fast twitch muscle of fish (Hochachka & Mossey, 1998) it has been proposed that a significant fraction of Cr (about 30 %) is not immediately available for the CK reaction in

these tissues. MR magnetization transfer experiments on skeletal muscle indeed revealed a pool of Cr that may be bound to matrix or cellular macromolecules, but this pool is in exchange with 'mobile', MRS-visible creatine and comprises only about 2 % of total creatine (Kruiskamp *et al.* 1999; Renema *et al.* 2002). The observation in human skeletal muscle that the methylene peak of Cr in ^{1}H MR spectra disappears during exercise (Kreis *et al.* 1999) further stimulated the discussion that a significant immobile Cr pool may exist, which is not readily accessible to creatine kinase. In another study a decrease in T_2 relaxation time of creatine methyl proton spins was observed during ischaemic fatigue in humans (Trump *et al.* 2001), suggesting reduced mobility of creatine. In contrast, ^{1}H MRS investigations of mouse skeletal muscle indicated that changes in Cr signals, observed immediately after death of the animal, mainly result from altered dipolar interactions related to removal of the phosphate moiety of this compound and not from changes in its cyto-architectural environment (in 't Zandt *et al.* 2000*b*). After eliminating the spectral appearance of these interactions by orienting the fibre direction of the muscle at 55 deg with the main magnetic field (magic angle), the Cr signal did not change in the early post-mortem phase. Interestingly, a recent study indicated that the electric charge of small molecules and proteins affects dipolar coupling interactions in skeletal muscle (Asslani *et al.* 2001).

Our quantification of total Cr content in adult mouse skeletal muscle, by ^{1}H MRS of muscles placed at the magic angle to avoid complications by dipolar interactions (Kreis *et al.* 1997), yielded similar values to those obtained by chemical means. In the GPS complex of animals at an age from 2 to about 6 months the intracellular total creatine concentrations were about 40 mM. This is close to the values published for type 2a/b muscles in mouse and rat (Kushmerick *et al.* 1992), which is what can be expected as the gastrocnemius muscle is dominating in this complex. Altogether these results do not point to a significant MR-invisible Cr pool (i.e. more than the error of the measurements, which is less than 10 %) in mouse skeletal muscle, as was already concluded for PCr in these muscles (Wiseman & Kushmerick, 1995). Further support for 'full MR visibility' of the total Cr pool comes from the observation that PCr/tCr ratios determined by ^{13}C MRS resemble the chemically determined values. Ischaemia experiments indicate that the MR-visible-PCr and free-Cr pools are in direct contact with each other in wild-type mouse skeletal muscle. Finally, the similarity of creatine levels in wild-type and mutant muscles as detected by ^{1}H MRS indicates that the mere presence of CK itself does not affect the MR visibility at rest of the Cr pool and hence its mobility.

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