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Cytoarchitectural and metabolic adaptations in muscles with mitochondrial and cytosolic creatine kinase deficiencies

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Abstract

We have blocked creatine kinase (CK) mediated phosphocreatine (PCr) ⇄ ATP transphosphorylation in mitochondria and cytosol of skeletal muscle by knocking out the genes for the mitochondrial (ScCKmit) and the cytosolic (M-CK) CK isoforms in mice. Animals which carry single or double mutations, if kept and tested under standard laboratory conditions, have surprisingly mild changes in muscle physiology. Strenuous *ex vivo* conditions were necessary to reveal that MM-CK absence in single and double mutants leads to a partial loss of tetanic force output. Single ScCKmit deficiency has no noticeable effects but in combination the mutations cause slowing of the relaxation rate. Importantly, our studies revealed that there is metabolic and cytoarchitectural adaptation to CK defects in energy metabolism. The effects involve mutation type-dependent alterations in the levels of AMP, IMP, glycogen and phosphomonoesters, changes in activity of metabolic enzymes like AMP-deaminase, alterations in mitochondrial volume and contractile protein (MHC isoform) profiles, and a hyperproliferation of the terminal cysternae of the SR (in tubular aggregates). This suggests that there is a compensatory resiliency of loss-of-function and redirection of flux distributions in the metabolic network for cellular energy in our mutants. (Mol Cell Biochem **184**: 183–194, 1998)

Key words: skeletal muscle mitochondria, creatine kinase, metabolic adaptation

Introduction

Creatine kinases (CK; EC 2.7.3.2) form a small family of mitochondrial and cytosolic isoenzymes which catalyse the reaction phosphocreatine (PCr) + ADP + H^+ ⇄ creatine (Cr) + ATP, a nodal event in the network for high energy phosphoryl transfer in vertebrates. Although many concepts have been formulated to explain CK's role in high energy-phosphoryl homeostasis [1, 2], relatively little is known about its involvement in maintaining the integrity of

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metabolic-energy compartmentalization [3–5] and the communication between intracellular sites of ATP consumption and production. Depending on cell-type one can find different CK isoforms in mitochondria (i.e. the dimeric or octameric sarcomeric (Sc) or ubiquitous (Ub) mitCKs) and cytosol (i.e. the homo and heterodimeric MM-, BM- or BB-CKs) in all tissues with large fluctuations in energy metabolism, such as muscle and CNS [2]. Individual CK family members may appear at different stages and locations during animal development but their functional activities appear usually coordinately at different cellular locations. It is generally assumed that CK's help in keeping subcellular levels of ATP and its hydrolysis products ADP, AMP, P_i and H^+ delicately balanced. In turn, these levels – or the ratios thereof – may govern the dynamic kinetics of many ATPases in different microenvironments [6–9] and may determine the rate of glycolysis and oxidative phosphorylation (OXPHOS) [10, 11] and the activity of ion-pumps ([2, 12, 13] and refs therein). The precise flux distribution through the CK reaction of high-energy phosphoryl metabolites in distinct mitochondrial, cytosolic or nuclear compartments is however very difficult to study as it is intertwined by high-energy phosphoryl interconversion reactions catalyzed by adenylate kinase (AdK) [14], nucleoside-mono and -di-phosphate kinases, hexokinase or glycerol kinase. Individual reaction steps in this elaborate and complex network might well be facilitated by the subcellular clustering of these kinases in multienzyme complexes [1], which may keep metabolites from entering the cellular pool. Interestingly, the allocation of CK's to distinct cellular sites is dynamically influenced in response to variation in energy demand and may be an important physiological mechanism(s) for regulating the enzymes' catalytic properties [2, 15].

In order to obtain a better understanding of the subcellular partitioning of components of the CK/PCr system, and to unravel its role in the compartmentalization of energy homeostasis in different cell types it will become of utmost importance to apply experimental methods which preserve the integrity of the cells delicate organization, and the communication between specific cellular 'aggregulons' or microcompartments. Fortunately, the rapid development of techniques to manipulate gene expression *in vivo*, in cell's and experimental animals, is providing us with powerful new tools for these kind of studies; Normal or mutant genes of interest can be either overexpressed, or their expression can be directed to specific tissues using 'conventional' transgenesis by micro-injecting DNA into the fertilized egg [16]. In an alternative approach, gene targeting in mouse embryonic stem (ES) cells creates the possibility to produce mice carrying predesigned mutations in the germline [17]. This technique, mostly applied as a gene 'knock-out' mutagenesis method, has already been used to generate hundreds of new mouse lines [18] and is currently one of the methods of choice for revealing unknown gene functions in the context of the whole animal (see [19–23] for review). We and others have applied the methodology for altering the expression levels of the different CK isoenzymes *in vivo*. Conventional transgenic techniques have been applied to direct B-CK and UbCKmit isoenzyme expression to mouse liver [24], as well as to induce ectopic B-CK expression in striated muscle tissue [25–27]. Our group has generated mice completely deficient in M-CK subunits and mice expressing reduced levels of M-CK, by gene targeting. The biological consequences of these mutations have been characterised [28, 29]. Likewise, animals lacking either the ubiquitous mitochondrial CK subform (UbCKmit), or the sarcomeric mitochondrial CK (ScCKmit) isoenzyme, with suprisingly little phenotypic effects, have been generated [30, 31]. Here, we summarize old and new data regarding the genotype-phenotype relationship in muscles of mice with single or combined M-CK and ScCKmit deficiency, under normal laboratory conditions. One particular intriguing observation, the developmental adaptation of metabolic and cytoarchitectural characteristics of muscle in response to mutation-induced defects, is discussed most extensively.

Nodal links between the CK-circuit and muscle (cell) physiology

In muscle, MM-CK mediated ATP production is mainly coupled to the local activity of SR- and plasma-bound Ca^{2+}-ATPases [6, 8, 9, 32], the Na^+/K^+-ATPase [33, 34] and the myosin ATPase involved in actin-myosin sliding during contraction in the sarcomeric M-band [35–39]. The MM-CK mediated reaction is also topologically coupled to the glycolytic metabolic reaction cascade in the I-band thin-filament region [35]. The ScCKmit reaction is thought to be involved in the transphosphorylation, channelling and transport of high-energy phosphoryl groups from mitochondria to the cytosol in muscle [40]. In addition, the ScCKmit mediated reaction may fuel mitochondrial ATPases (Ca^{2+} or K^+-ATPases), in analogy to the cytosolic situation, but this is still a rather hypothetical possibility.

CK-mutant mice

To assess the effects of CK absence on these different functions in muscle we have generated mice lacking either the mitochondrial CK (ScCKmit), the cytosolic CK (M-CK) or a combination of both enzymes, by interbreeding ScCKmit [–/–] [13, 30, 31] and M-CK [–/–] [28] deficient mice in all possible combinations. By genotyping 215 offspring from intercrosses between double heterozygous (i.e. ScCKmit [+/–]; M-CK [+/–]) animals eleven wild-types

and ten ScCKmit [–/–]; M-CK [–/–] double mutants were identified. Contrary to expectation, these double deficient mice (henceforth indicated as CK [–/–]) were not overtly different from wild type, or single mutant animals, and bred normally. Zymogram assays of cardiac and skeletal muscle extracts of 6 week old CK mutants confirmed that the anticipated CK profiles were present in the ScCKmit and M-CK single and the CK double mutants muscles (Fig. 1). No ectopic expression of any of the two other members of the CK gene family, brain-type (B-CK) or ubiquitous mitochondrial CK (UbCKmit) was seen in any mutant. Only in heart muscle we saw a slight increase in the level of BB-CK isoenzyme but this can be explained by the lack of capture of B-type subunits in MB-CK heterodimers as noted before [28]. Residual total CK activity in heart and skeletal muscle extracts was lowered to 0.3 and 2% of wild-type levels, respectively, and can be entirely attributed to the BB-CK and UbCKmit content, which in skeletal muscle is found especially in capillary or vasculature endothelium and satellite cells. Despite the fact that adenylate kinase (AdK) activity was inhibited by the inclusion of P1,P5-(di(adenine-5′)-pentaphosphate (ApSA) in our zymogram assays often a weak band of AdK activity was seen in the extracts from double knock-out animals. Although many explanations are possible this may suggest that AdK activity is increased in CK [–/–] mice, consistent with the idea that there is functional overlap and interrelated coupling between the CK and AdK circuits in the OXPHOS and glycolytic networks for storage and transport of high energy phosphoryl groups [41]. Currently, in collaboration with Dr. N. Goldberg, we are studying this phenomenon in more detail by the use of ^{18}O incorporation into the β and γ phosphoryls of ATP and the phosphoryl groups of various other metabolites in the structurally intact tissues.

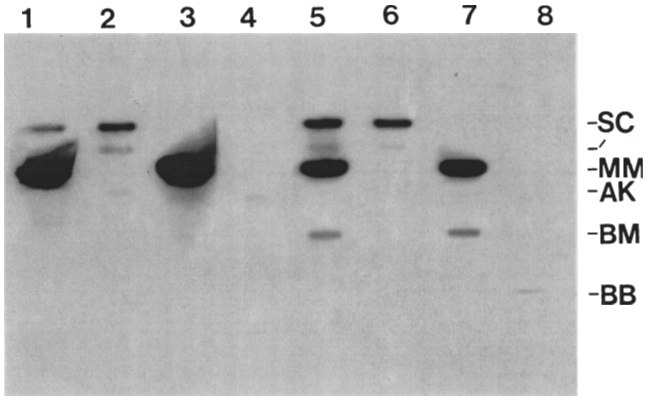

Fig. 1. Zymogram gel analysis of skeletal (lanes 1–4) and cardiac (lanes 5–8) muscle extracts from wild-type (+/+) (lanes 1, 5), M-CK [–/–] (lanes 2, 6), ScCKmit [–/–] (lanes 3, 7) and CK [–/–] double mutant (lanes 4, 8) mouse. Note the absence of the ScCKmit and MM-CK isoforms in the different mutants and note also the weak signal of residual AdK activity (not fully inhibited by Ap5A) in the CK [–/–] mutant.

Metabolite levels

Chemical methods were used to examine whether CK deficiency had influenced the levels of energy-related metabolites in muscles at rest [13, 30, 31]. Concentrations of ATP in CK [–/–] muscles (18.1 ± 2.6 μM/g dry wt) were somewhat lower than in wild-type and ScCKmit [–/–] muscles (24.5 ± 4.8 and 22.9 ± 2.0 μM/g, respectively), but only marginally lower than in M-CK [–/–] muscles (19.1 ± 2.1 μM/g). Another series of measurements showed that AMP and IMP levels (0.51 ± 0.08 or 0.66 ± 0.10 μM/g, respectively) in the gastrocnemius-soleus-psoas complex of M-CK [–/–] and CK [–/–] mutants were roughly 5-fold higher than the levels in wild type and ScCKmit mice (0.11 ± 0.03 or 0.15 ± 0.04 μM/g, for AMP or IMP, respectively). It is important to note that any direct estimates on the primary effects of CK absence on the degree of overproduction of AMP and IMP may be unreliable, as adaptation in the primary metabolism of these compounds, attributable to other enzymes in the adenylate metabolic network, may involve significant redirection of flux distribution (see below).

Most surprisingly, we observed that the concentration of PCr in the medial gastrocnemius of CK [–/–] animals was only slightly lower than in wild-types (51.6 ± 5.2 vs. 62.1 ± 2.2 μmoles/g dry wt). Levels of total Cr (Cr + PCr) were similar in all muscles (with non normalised values ranging between 86.2–106.5 μmoles per g dry wt), indicating that Cr import mechanisms *per se* are not affected. This unexpected presence of PCr was confirmed by *in vivo* ^{31}P nuclear magnetic resonance (NMR) spectroscopy (Fig. 2). Fully relaxed resting state spectra of CK [–/–] hindlimb muscles showed a clear PCr peak, appearing at exactly the chemical shift position expected for PCr (at 2.44 ± 0.02 ppm from the position of γATP). The relative positions of the three ATP resonances in double mutant skeletal muscle spectra were similar to wild-type spectra. Relative peak areas (as % of total) of energy metabolites in wildtype, ScCKmit [–/–] and M-CK [–/–] single mutants were all similar [13], but CK [–/–] mutant muscles showed a significantly lower PCr signal (35.6% vs. 46.5–47.3%) and somewhat higher ATP signals with respect to the total phosphate signal area. Consequently, this results in a 30% lower PCr/ATP ratio. We think that NMR data give a better reflection of the actual metabolite levels than the chemical method, because minor energy metabolite changes can be induced by the freezing procedure. In another report [42] we have provided evidence that the PCr pool does not exchange with ATP and is metabolically completely inert, even under conditions of complete hypoxia. This leaves the question how and when

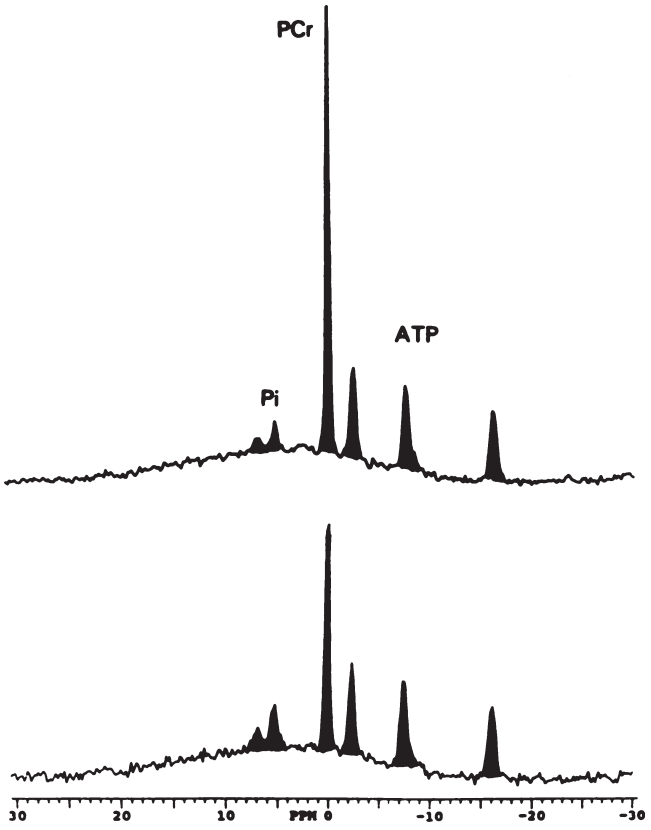

Fig. 2. ^{31}P-NMR recordings of phosphate metabolites in lower hind limb musculature at rest of wild-type and CK [–/–] mice. Spectra in the top panel (wild type) and the bottom panel (CK [–/–]) represent the average of 48 free induction decays of 70° pulses and 5 sec repetition time, giving a time resolution of 4 min per spectrum. Positions of signals from the phosphoryl groups in PCr, the γ, α- and β- groups in ATP (from left to right), the inorganic P_i, and the phosphomonoesters (outermost left signal) can be seen as separate peaks.

the PCr was formed in our mutants and whether it can accumulate in a time-dependent fashion during development or growth. At this moment it is even not clear if PCr was imported via circulation or via gap-junctional contact with cells from muscle-adjacent tissues, or formed by phosphoryl-transfer-enzymes with low substrate specificity.

The peak area measurements also show that relative levels of inorganic phosphate (P_i) and phospho-mono-esters (PME) have increased significantly, about 2–3 fold, in CK [–/–] muscles, more than in the muscles with single CK mutations. The abnormal accumulation of phospho-mono-esters points to a deregulation or adaptation of the flux through the glycolytic pathway, which however does not lead to significant accumulation of lactate. We come to this conclusion because pH calculations from the res–onance positions in the ^{31}P spectra yielded similar values for wild-type (7.29 ± 0.07) and CK mutants muscles at rest (7.17 ± 0.05 for ScCKmit [–/–], 7.24 ± 0.02 for M-CK [–/–] and 7.21 ± 0.05 for CK [–/–]). Currently, in order to better understand the basis and origin of the phospho-monoester accumulation we are using high resolution NMR spectroscopy of metabolites in a perchloric acid extract of clamp frozen muscle to identify the precise chemical origin of the signals.

Earlier, we had reported that resting-state glycogen content and the ability to consume this glycogen was about 60% increased in M-CK muscle compared to wild-type [28] mainly due to an increase in fast-type fibers. A similar increase was found for CK [–/–] double mutants, but ScCKmit [–/–] animals had near wild-type glycogen levels [13]. Taken together our data suggest that CK absence causes a considerable re-direction of flux distribution in various branches of primary metabolism. This may lead to compensatory adaptation in the steady-state levels of AMP, IMP and inorganic phosphate as well as the levels of phosphomonoester carbohydrate-metabolites. It should be emphasized again that all muscles studied were from animals kept under normal housing conditions. How the steady-state metabolite levels can fluctuate during development, growth and activity-induction of the different CK-mutants is subject for further study.

Physiology and cytoarchitectural adaptations

Details on the physiological consequences of CK ablation have been published [13, 31, 42]. All observations relate to the testing of muscle performance upon artificial electrical stimulation, *ex vivo*. From our studies, as illustrated in Fig. 3 we had concluded that M-CK is the dominant governing principle in the reduction of tetanic force output (i.e. generation of burst activity; [13, 28]) in skeletal muscle. If combined with M-CK deficiency, but not singly, ScCKmit deficiency leads to increase in relaxation time [42].

In a first *in vivo* survey we found no overt effects on animal activity or behaviour, in keeping with the fact that a number of biochemical parameters (in f.e. heart mitochondrial activity, oxygen consumption) were essentially unchanged [13]. Apparently, muscle physiology is not challenged to threshold levels under normal laboratory housing conditions, but we might have missed subtle effects. Therefore, to examine the basic physiological functions in our mutants in somewhat more detail we started computerized telemetric analyses [43, 44] of heart beat rate, core temperature and gross locomotor activity. Figure 4 shows the typical recordings of one CK [–/–] double knock out and one wild type animal during a continuous monitoring period of 2 days with successive light (14 h) and dark (10 h) intervals. After averaging the measurements on three CK [–/–] and two wild type animals there were no significant differences. Average body temperature ranged from 36.7°C (dark period) to 35.9°C (light period) in mutant and wild type animals, heart

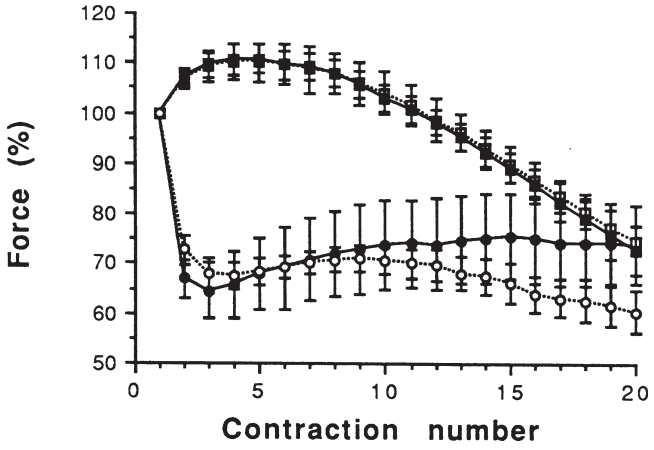

Fig. 3. Contractile characteristics of wild-type and CK-mutant medial gastrocnemius muscles during high-intensity exercise. The isometric force production profiles (as % of the initial force) in wild-type (open squares), ScCKmit [–/–] (black squares), M-CK [–/–] (black circles) and CK [–/–] double-mutants (open circles) in muscles are shown. The muscle complex was stimulated 4 times per sec at 100 Hz with an intermittent stimulus duration of 165 msec; the brief periods between contractions were sufficient for complete relaxation. Note that the ScCKmit force profile overlaps with the wild type profile. Recovery, as seen in the M-CK [–/–] profile is absent in the CK [–/–] profile.

beat rates varied between average 469–521 (light-dark) in CK [–/–] mice to average 513–561 (light-dark) beats/min in wild types. Locomotor activity varied between 44 (dark) to 26 (light) for CK [–/–] and 57 (dark) to 27 (light) crossings/min for wild type. Although these figures suggest that CK [–/–] mice may be slightly less active during the dark period, the differences are subtle and obviously many more recordings on a large series of animals are necessary to make this statistically significant. As is evident from Fig. 4 the fluctuations in minima and maxima of body temperature, heart beat rate and activity within individual dark or light intervals were considerable. We therefore limited ourselves to obtaining a qualitative impression because of several technical and logistic reasons. One reason being that the current state of sophistication of the equipment is still rather limited and in combination with the 'difficult-to-standardize surgical transplantation-location' of the transmitter and electronic leads can be the cause of considerable experimental variation. Another – more interesting – variable can be introduced by the genetics of the knockout procedure, as all our mutants and controls have a mixed and variable background of 129/Sv × C57BL/6 inbred genes. There is tight coupling of brain function to all three variables measured (heart rate, temperature and activity) and it is now becoming increasingly clear that effects of genetic background cannot be ignored in these studies [45]. Interestingly, both groups of mice show the normal physiological response towards light and dark periods. We thus may conclude that CK absence does not form a serious obstacle to normal heart and skeletal muscle physiological-functioning under laboratory conditions.

Consequences for mitochondria and ER membranes

Previously, we had observed that M-CK absence is compensated by an adaptation in mitochondrial volume in type 2 (fast) fibers of the gastrocnemius-psoas-soleus (GPS) complex of M-CK [–/–] mice, but that the mitochondrial ultrastructure is otherwise fully normal [28, 29]. To see whether other compensatory mechanisms were involved in ameliorating the complete loss of the Cr-PCr shuttle function in CK [–/–] animals we repeated the morphometric inspection and examined the fiber ultrastructure of diaphragm, heart, intercostal, gastrocnemius and soleus muscles. Although we did not quantify the effect in absolute terms, the enlarged intermyofibrillar mitochondrial volume (1.5–2 fold) was clearly apparent in all muscles, except heart [13, 31]. Biochemical activity determination showed an increase from 353 ± 76 to 470 ± 42 mU/mg protein for COX and from 69 ± 15 to 117 ± 21 mU/mg protein for CS (both mitochondrial marker enzymes) in 600 g whole-tissue extracts from hindlimb. In contrast, COX and CS levels in heart (955 ± 303 vs. 937 ± 354 for COX and 236 ± 57 vs. 263 ± 86 for CS, respectively) did not differ between wild type and CK [–/–] mice, in keeping with the ultrastructural analyses. Mitochondria in CK [–/–] skeletal muscles were often packed in rows and their sizes were highly variable, ranging from extremely large (more than 5 μm in length) to very small. Moreover, mitochondria-rich fibers of all CK [–/–] muscles, including heart, diaphragm and large glycolytic skeletal muscles, were distinctly different from other mutants and wild type in that they contained large(r) numbers of lipid droplets [13]. In semi-thin 1 μm sections, these lipid droplets can often be seen as strings of beads. Electron microscopic examination revealed the lipid droplets to be located immediately adjacent to, and occasionally inside, the intermyofibrillar mitochondria. Association to the subsarcolemmal mitochondrial population was only rarely observed. Furthermore, mitochondria containing glycogen, lipofuscin granules, and other lysosomal structures were present in increased numbers in CK [–/–] muscles (data not shown). These deposits are common to diseased muscle and occur to a much lesser extent in normal muscle during ageing [46]. Are these changes cause or consequence of an altered mitochondrial physiology or is a cytoplasmic factor the principal governing principle? To address these questions we next examined whether the number of mitochondrial contact sites had undergone changes in double CK mutants (Fig. 5A). Contact sites are fusions between the inner and outer mitochondrial membranes which are dynamically regulated multi-subunit structures that assemble in coordinance with

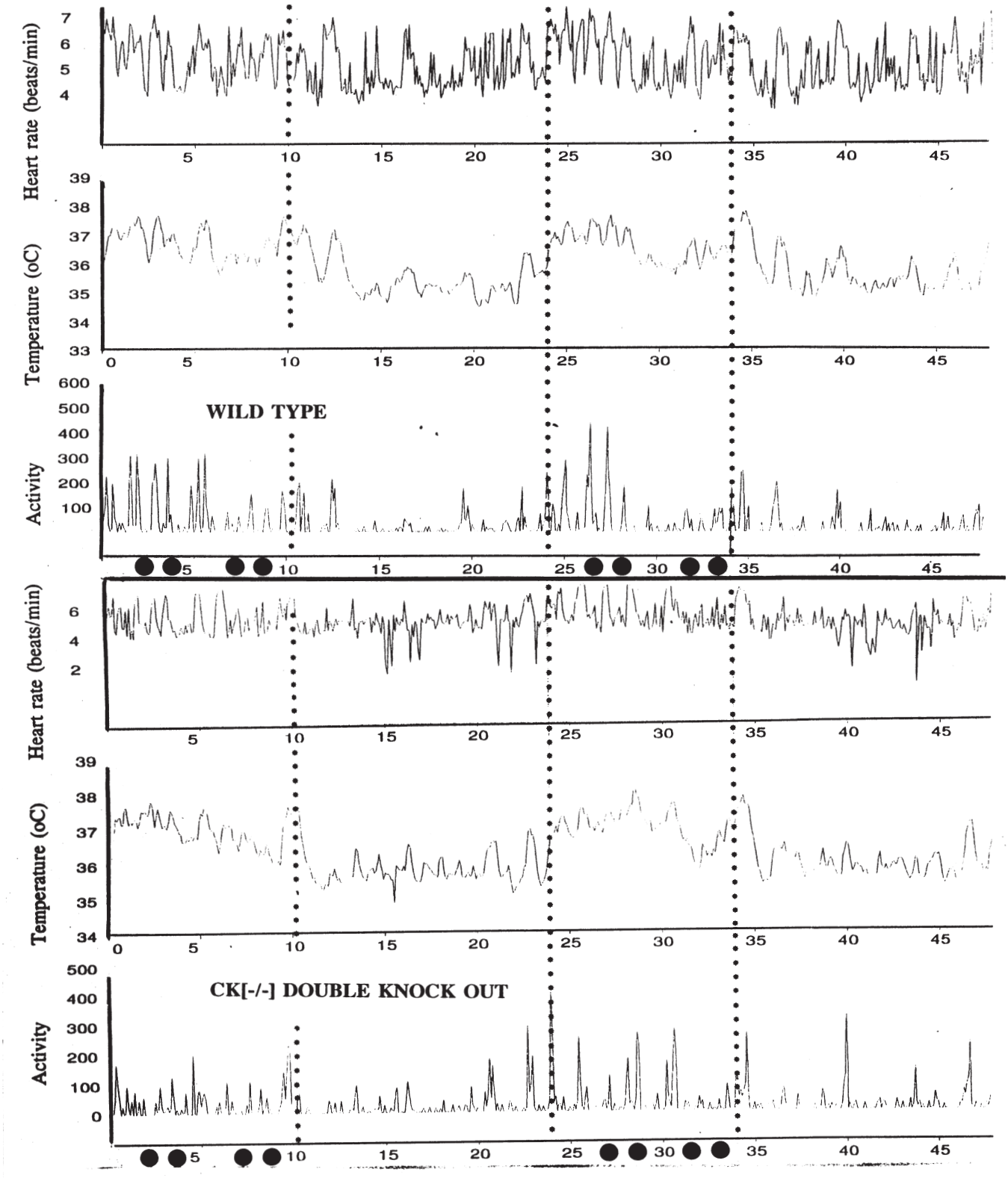

Fig. 4. Representative telemetric recordings of heart rate (top panels), body temperature (°C, middle panels) and locomotor activity (bottom panels) in one single wild type and CK [–/–] animal. Intermittant periods with dark (10 h; black dots) and light (14 h) housing conditions are indicated. Note the (normal) drop in body temperature and activity during the light period in both animals.

metabolic activity [14, 47, 48]. A direct function for mitCK in the assembly of these structures has been suggested by the extensive studies of Wallimann and coworkers [40, 15] showing that mitCK-octamers may physically bridge the gap between the mitochondrial inner- and outer membrane. For contact site surface density measurement we cytometrically compared [49] skeletal and myocardial muscles of wild type and CK [–/–] mutants. Suprisingly, even though different metabolic pathways are used (as described above) we found no significant difference in contact site densities (Ss) between wt (0.37 ± 0.05) and CK [–/–] (0.36 ± 0.02) animals in femoral quadriceps muscle. The data for myocardial muscle were Ss = 0.31 ± 0.01 and Ss = 0.36 ± 0.02 for the wt and CK [–/–], respectively. The slight increase seen in CK [–/–] mitochondria - if real - may originate from the fact that CK [–/–] mice are more affected by emotional stress upon handling, prior to anaesthetizing the animals. The larger S.E.M. value for skeletal muscle is due to the less efficient fixation. Our data indicate that the formation of contact sites *per se* is not blocked by the absence of ScCKmit in our mutants and we are currently investigating the dynamics of contact formation under regimes where we stimulate or inhibit OXHPOS, to examine whether CKmit has any role at all in the dynamic process of contact site formation.

Another distinct feature of CK [–/–] animals was the omnipresence of conspicuous, darkly staining, elongated inclusions within the large fibers of gastrocnemius and intercostal muscles (Fig. 5B). Within these fibers, the inclusions are seen at both subsarcolemmal and intermyofibrillar localizations. Ultrastructural examination of various fibers revealed that the inclusions consist of closely packed, longitudinally oriented clusters of membranes, known as tubular aggregates (TAs). Besides the well-ordered aggregates, unorganized, more dilated membrane structures with varicosities containing electron-grey material were also observed. TAs are a distinct pathological structure in skeletal muscle consisting of aggregated terminal cisternae or longitudinal components of the sarcoplasmic reticulum (SR) [46]. They may be functionally equivalent to hypertrophy of the SR terminal cisternae and have been shown to be highly reactive with antibodies against Ca^{2+}-ATPases and calsequestrin. The maximal calcium content of TA-containing fibers is increased [50]. Remarkably, TA-like structures were not found in single mutations and have never been reported in creatine-depleted muscles, when rodents are fed with creatine analogues [2]. In humans, the occurrence of TAs has been associated with conditions caused by mutations in genes for the dihydropyridine receptor subunits, the ryanodine receptor (Ca^{2+}-channels), the adult sodium channel gene SCN4A [46, 51, 52], and a variety of other muscle diseases [53]. In mice, TAs have been observed in murine dystrophy heterozygotes [54] and in congenic mice

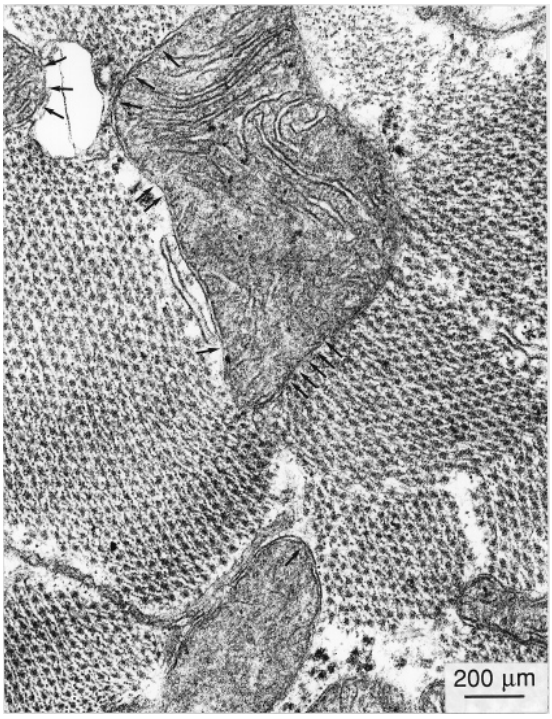

Fig. 5A. Electron micrograph of a transverse section of a heart muscle cell. The right side of the surrounding membranes of the mitochondrion at the top are obliquely cut. Contact sites are designated by arrows.

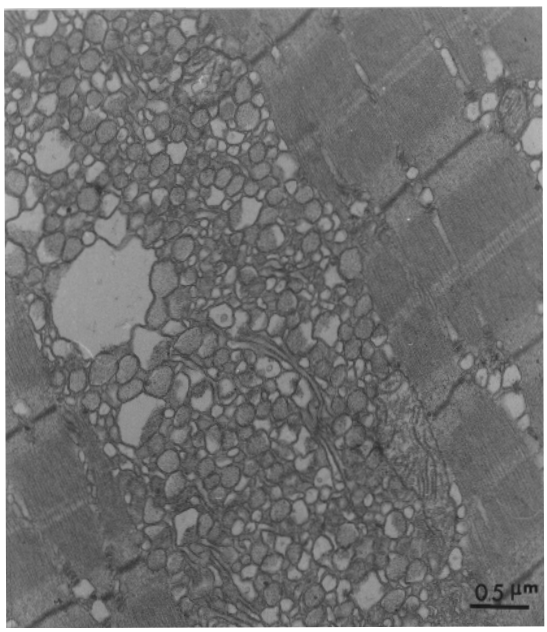

Fig. 5B. Cell-morphological abnormalities and the formation of tubular aggregates in CK [–/–] muscles. Shown is an electron micrograph revealing the different aspects of a section chosen from a darkly toluidine-blue stained area from CK-deficient gastrocnemius muscle. Note the hyperproliferation of tightly packed, longitudinally oriented tubes of the SR (doe more detailed analyses see [42]).

of the MRL +/+ substrain [55]. From study of the latter model it is clear that appearance of TAs is genetically predisposed, and gender (i.e. hormone) and age related. In our double mutants we observed that terminal cisternae of the SR, which are located in the direct surroundings of, or connected with tubular inclusions, were heavily dilated. This suggests that the TAs in our CK [–/–] muscles are indeed derived from the SR. The myofibrillar compartment adjacent to the tubular aggregates showed no structural abnormalities, and triads in those myofibrils also appeared normal. TAs are clearly a manifestation of disease, and almost certainly a secondary adaptation to the abnormal cell physiological responses evoked by CK absence. Evidently, they develop already under normal laboratory conditions, and it will be interesting to see whether their formation interferes with normal development, and in turn may affect muscle strength or even cause disuse of muscle, especially if animals are kept under regimes for higher activity, involving more strenuous conditions for muscle labour.

Enzymatic adaptations

To explain the changes in contractile performance of M-CK [–/–] and CK [–/–] muscles one could postulate either direct effects of CK absence or invoke (secondary) perturbations in the metabolic network for production and use of high-energy phosphoryls. This may involve changes in enzymatic activities, concentrations, or both (see [56] for a review on 'metabolic network flexibility'). As analyses of metabolic flux distributions are intrinsically difficult we performed only a merely random survey of activities of nodal enzymes in the network directly coupled to the CK reaction. Mutant skeletal muscle fibers may temper decreases in the free energy of ATP hydrolysis by the transphosphorylation of free ADP to ATP and AMP through the action of adenylate kinase. Due to the near-equilibrium nature of this reaction, net forward flux can be sustained by increasing substrate (i.e. free ADP) or decreasing product (free AMP) concentrations. AMP formed by the adenylate kinase reaction can be de-aminated by the enzyme AMP deaminase, a tetrameric enzyme composed of identical 80 kD subunits that catalyzes the non-equilibrium deamination of AMP to IMP and ammonia. IMP is largely retained within the myocyte until subsequent reamination to AMP via the purine nucleotide cycle [57]. The AMP deaminase reaction is thought to be controlled, at least in part, by AMP concentration, allosteric modulators such as ADP and inorganic phosphate and ATP turnover associated with muscle contraction [58]. As many of these parameters are affected in CK-mutant muscles we studied the activity and structure of the enzyme in our mouse models. In hindlimb muscle composed of a mixture of fiber types, AMP deaminase activity assayed under optimal in vitro conditions [59] was decreased ~60% in the M-CK deficient versus wild-type muscle. When examined at low AMP concentrations (< 0.2 mM), AMP deaminase from M-CK deficient mice displays a marked increase in affinity coupled with a decreased V_{max} compared with the enzyme from wild type muscle. This kinetic behaviour is characteristic of negative cooperativity that can be exhibited by oligomeric enzymes and is accompanied by changes in the apparent subunit molecular weight determined with SDS-PAGE and Western blots. In muscle from wild-type mice, about 80% of the immunoreactive protein was found to have an apparent molecular mass of 80 kD. In contrast, mixed muscle from the M-CK deficient had less than half as much 80 kD AMP deaminase protein coupled with an accumulation of smaller species with apparent molecular weights of 60 and 56 kD (data not shown). This loss of 80 kD AMP deaminase is consistent with the decline in *in vitro* activity. Interestingly, these changes in molecular weight and enzyme activity are also found in β-GPA treated rats [60] and may be related to post-translational changes such as proteolysis or covalent modifications. How the overall-purine metabolism in functionally intact muscles, during rest and during intense exercise, is influenced by the loss of CK isoforms and the secondary changes in AMP deaminase structure and activity, remains to be determined.

We postulated earlier that also at the ATP-consumption sites in the metabolic network adaptive changes in the profiles of enzyme-activities could be involved. Suprisingly, however, biochemical assay of whole muscle extracts showed that neither the activity nor the concentration of SR Ca^{2+}-ATPases was conspicuously different between mutants and wild type animal. SR Ca^{2+}-ATPase activity (expressed as mU/mg protein; n = 3 animals each) was 76.5 ± 9.5 in wild type, 75.5 ± 9.8 in M-CK [–/–] and 76.6 ± 10.0 in CK [–/–] mutants. SR Ca^{2+}-ATPase content ranged between 94.3 ± 11.0 and 95.2 ± 11.8 pmol/mg protein in these animals. We feel that in addition to these measurements our studies should include the analyses of SERCA- and PMCA-isoenzyme profiles, to rule out that isoenzyme switches are involved. This is a goal for the near future. As myosin-ATPase is the other principal determinant of energy demand in working muscle and alterations in myosin isoenzyme profiles could result in shifts in intrinsic ATPase activity, shortening velocity, and energetic economy we also studied the myosin heavy chain isotype (MHC) distribution [61, 62]. Comparison of the MHC patterns in the representative slow soleus muscle, intermediate type gastrocnemius and diaphragm, and fast psoas and extensor digitorum longus (EDL) muscle, showed that transitions in isomyosin heavy chain types do not occur in any of these types of muscle in ScCKmit [–/–] or, more strikingly, CK [–/–] mice. In contrast, small but significant changes became apparent in fast muscles from M-CK [–/–] mice. Profiles in M-CK [–/–] EDL, psoas and

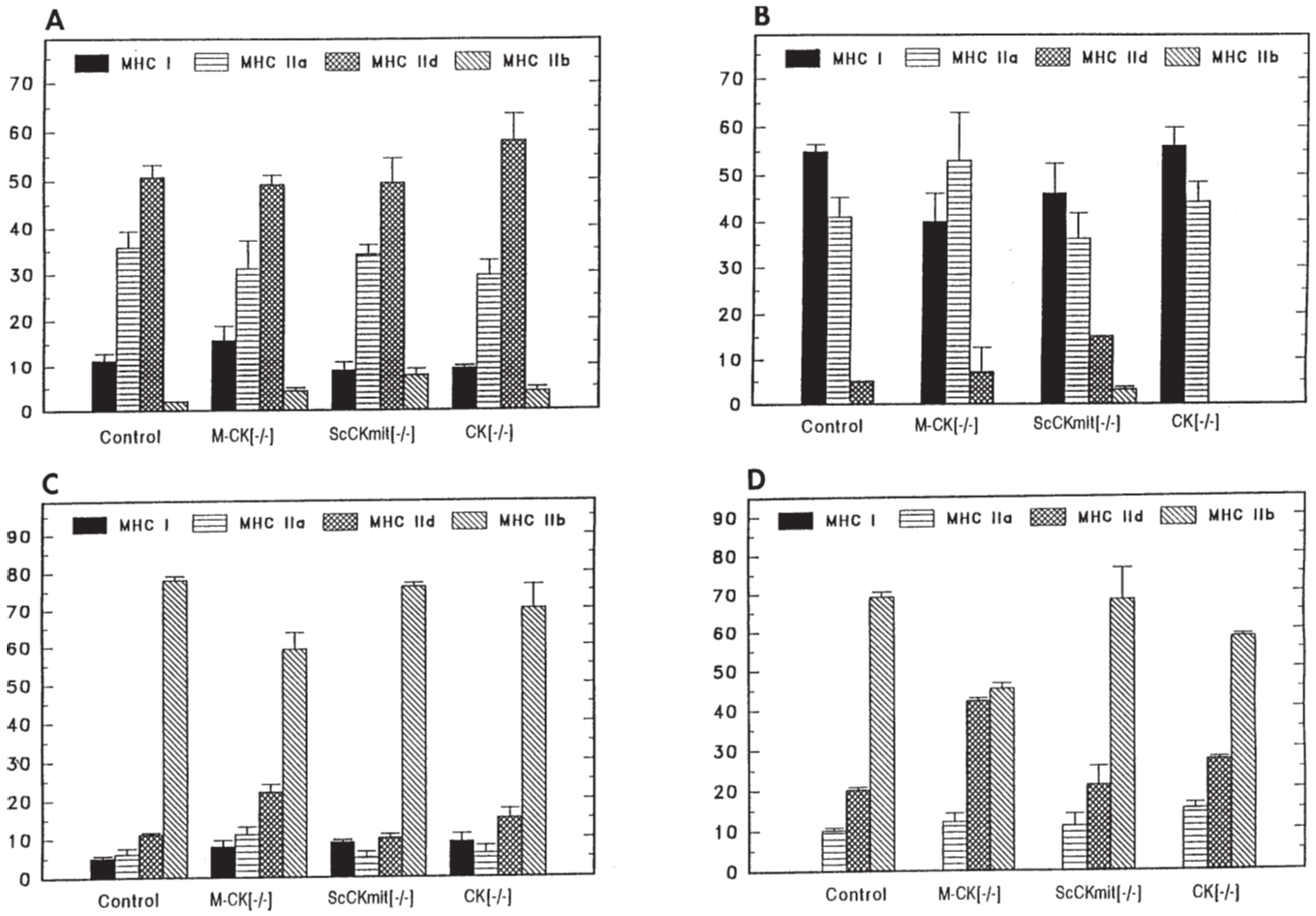

Fig. 6. Diagrams of myosin heavy chain (MHC) distribution in extracts from several muscles of a wild-type, ScCKmit [–/–], M-CK [–/–], and CK [–/–] mouse. Electrophoretic MHC separations of (A) diaphragm, (B) soleus, (C) gastrocnemius, and (D) psoas muscle extracts were densitometrically scanned. Figures at the left indicate the levels of specific MHC isoforms as a percentage of total (100%). Every bar represents three gels from one muscle. Note that the shift in the MHC-profile is most prominent in M-CK [–/–] animals.

gastrocnemius display a slight fast-to-slow shift, with 40–50% increase in MHCIId and 20–25% decrease in the MHCIIb content, compared to wild-type and ScCKmit [–/–] and CK [–/–] mutants (Fig. 6). Changes in MHC in cardiac atria and ventricles were not observed for any of the mutants. As judged from the (rather small) magnitude of changes we expect the involvement of the MHC-ATPase isotype to play only a minor role in altering muscle performance, perhaps more so in the relaxation and long-term performance of skeletal muscle, aspects which indeed differ between CK [–/–] and MCK [–/–] mutants. Obviously, under normal laboratory conditions there is no stringent selection on these aspects of muscle physiology.

At this point we would like to conclude that the deviation from the situation in wt in our mutants can be most easily explained as a direct effect of CK loss on (local) ATP. As many Ca^{2+}-ATPases operate close to thermodynamic equilibrium, this may have immediate effects on $[Ca^{2+}]_i$ levels in muscle and evoke a cascade of secondary cellular events [42]. In addition, the ATP supply for the myosin ATPase cycle and actomyosin sliding may be somewhat perturbed in our mutants. Importantly, pleiotrophic compensatory cytoarchitectural and enzymatic changes may obscure the phenotypic consequences, and do so in a mutation-type and fiber-type dependent manner. In any future studies of thresholds and energy networks, particularly during development of our CK mutants, we must therefore focus on obtaining a fully detailed picture of this background. This is a challenging and labourious endeavour but the knowledge may ultimately help us to discriminate between direct consequences of ablation of CK functions and indirect effects which most likely represent the fraction of phenotypic functions that cannot be compensated by other genes and pathways.

Materials and methods

Generation and genotyping of CK deficient mice

Procedures involved in the construction of the different targeting vectors, transfection of wild-type ES cells and the generation of M-CK [–/–] and ScCKmit [–/–] nullizygous mice have been described in detail elsewhere [13, 28, 31, 63].

In vitro *and* in vivo *measurement of CK isoenzyme and mitochondrial enzyme activities and metabolite levels*

Zymogram analyses and measurements of mitochondrial indicator enzyme activities (cytochrome *c* oxidase and citrate synthase) were done as described previously [13, 28, 31, 42]. Procedures for the extraction and concentration measurements of creatine and adenosine metabolites have been described [31]. *In vivo* ^{31}P-NMR spectra of mouse hind limb muscles at rest were recorded on an Oxford Instruments magnet (4.3 T), equipped with a S.M.I.S. spectrometer, and working at 73 MHz. Probe characteristics and experimental conditions were essentially as described previously [28, 31, 64, 65]. pH values were calculated from the chemical shift of the P_i signal as described previously [64, 65].

Physiological measurements of skeletal muscle function

Contractile characteristics and force measurements of medial gastrocnemius muscles were performed as described in detail elsewhere [66, 13, 31] and further assessed using three tetani (duration 150 msec; stimulation frequency 100 Hz). Force signals were digitized (1000 Hz) and analyzed for peak force, time to peak force, and half time of relaxation (time for force to fall from half to a quarter at the end of stimulation [67]). Telemetric monitoring of heart beat rate, body core temperature and gross locomotor activity was performed with the use of a telemetry system connected to a computer data acquisition program (Data Sciences, St. Paul, MN, USA). After intraperitoneal surgical implantation of a transmitter (TA10ETA-F20) mice were allowed to recover for 21 days, during which the mice regained their initial, pre-surgical weight. Monitoring was performed during two successive days with light/dark periods of 14/10 h as described [43, 44].

Light microscopical, ultrastructural and biochemical fiber typing

Cross sections of the gastrocnemius-plantaris-soleus (GPS) muscle groups were stained according to standard histochemical procedures as described [28]. Sample preparation, pre-and post-fixation and Epon embedding was exactly as described [28]. Ultrathin sections, double contrasted with uranyl acetate, were examined in a Philips electron microscope EM 301 or a JEOL TEM 1010. Details of procedures used in the morphometric and qualitative aspects of mitochondrial contacts sites will be published elsewhere (Verdoodt *et al*. submitted). For analysis of myosin heavy chain (MHC) composition, crude extracts from individual muscles from a wild-type, ScCKmit [–/–], M-CK [–/–], and CK [–/–] double mutant animals were prepared and electrophoretic profiles on gradient polyacrylamide gels were evaluated as described [68].

SR Ca^{2+}-ATPase determinations

The procedures for determining activity and concentration of SR Ca^{2+}-ATPase (measured by Ca^{2+}-dependent ATP hydrolysis and steady-state phosphorylation) have been described elsewhere [42].

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