Skeletal muscle adaptation in response to voluntary running in Ca\(^{2+}\)/calmodulin-dependent protein kinase IV-deficient mice

Takayuki Akimoto, Thomas J. Ribar, R. Sanders Williams, and Zhen Yan

Departments of 1Medicine and 2Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, 3Department of Life Science, Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Tokyo 305-8902, Japan

Submitted 21 May 2004; accepted in final form 23 June 2004


ADULT SKELETAL MUSCLES CONTAIN several subtypes of myofibers that differ with regard to metabolic profile, contractile property, and susceptibility to fatigue (4, 29). The fiber type composition is established during embryonic and postnatal development (15, 25). Adult skeletal muscles are capable of adaptation in response to altered functional demands, such as endurance exercise, involving orchestrated signal transduction from neuromuscular activity to the genetic regulatory machinery (23). However, the molecular mechanisms responsible for the maintenance of slow muscle phenotype and exercise-induced skeletal muscle adaptation remain to be fully elucidated.

Previous studies showed that Ca\(^{2+}\) signaling plays an important function linking different patterns of motor nerve activity to distinct programs of gene expression that establish phenotypic diversity among skeletal myofibers (5). Investigators at our laboratory (34) hypothesized that sustained increases in regulatory pools of intracellular Ca\(^{2+}\) as a result of tonic patterns of motor neuron activity stimulate the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase calcineurin, leading to increased transcription of genes expressed selectively in fatigue-resistant skeletal myofiber types (types I and IIA).

Subsequent investigations provided evidence that calcineurin-dependent signals are transduced by transcription factors that include nuclear factor of activated T cells (NF-AT) and myocyte enhancer factor 2 (MEF2) to relevant target genes and are amplified by concomitant activation of other Ca\(^{2+}\)-regulated signaling cascades that can be initiated by Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK) activities (13). The identity of the CaMK involved in this process has not been identified, however, and it could be any of the multifunctional CaMK, such as CaMKI, CaMKII, or CaMKIV.

Along this line of investigation, investigators at our laboratory (32) have reported that forced expression of a constitutively active form of CaMKIV in murine fast-twitch skeletal muscle results in enhanced mitochondrial biogenesis and increased types I and IIA fibers. These phenotypic changes are associated with increased mRNA and protein expression of proliferator-activated receptor \(\gamma\)-coactivator 1\(\alpha\) (PGC-1\(\alpha\)), a pivotal regulatory protein in skeletal muscle fiber type specialization and mitochondrial biogenesis (10, 19). This finding suggests a possible link between CaMKIV activity and PGC-1\(\alpha\) gene regulation, which is supported by a later finding that Ca\(^{2+}\)-regulated PGC-1\(\alpha\) protein expression in differentiated myotubes is dependent on CaMK activity (12). Other studies have demonstrated that muscle contractile activity induces PGC-1\(\alpha\) mRNA and protein expression (2, 18, 26) and that overexpression of PGC-1\(\alpha\) in fast-twitch skeletal muscle is sufficient to increase mitochondrial density and percentage of types I and IIA myofibers in a transgenic mouse model (10). Collectively, these findings support the notion that a CaMK, possibly CaMKIV, plays an important role in fiber type specialization and maintenance and in exercise-induced genetic reprogramming in skeletal muscles.

In light of the above findings, it is important to establish which CaMK proteins are expressed in mammalian skeletal muscle. The published findings for CaMKIV are inconsistent. In two separate reports, CaMKIV protein was reported not to be readily detected using immunoblot analysis in human and murine skeletal muscle (22, 32), while in another study,
CaMKIV was reported to be expressed in murine skeletal muscle and further induced by energy deprivation (40). On the other hand, CaMKII autonomous activity (independent of Ca²⁺ and calmodulin) has been shown to be increased in contracting skeletal muscles (6, 22), suggesting a potential functional role for CaMKII in mediating the genetic events in skeletal muscle adaptation. Thus it is necessary to determine unambiguously the dependence of skeletal muscle adaptation on specific CaMK activities in response to acute and long-term exercise training.

An intriguing finding from a recent study brought additional attention to the possible dependence of mitochondrial biogenesis and fiber type switching on CaMKIV activity. Zong et al. (40) reported that energy deprivation increased CaMKIV and PGC-1α protein expression as well as activation of the endogenous AMP-activated protein kinase (AMPK) and enhanced mitochondrial biogenesis in murine skeletal muscle; all of these were blocked by forced expression of a dominant-negative form of AMPK. Previous studies clearly linked increased CaMKIV activity to mitochondrial biogenesis (3, 20, 31, 39), at least partially through the activation of transcription factors such as nuclear respiratory factor 1 (3). Zong et al.’s (40) findings suggest that CaMKIV is expressed in skeletal muscle and that elevated expression of CaMKIV upon AMPK activation in skeletal muscle, which stimulates PGC-1α expression, mediates mitochondrial biogenesis in response to energy deprivation. An important question is whether CaMKIV activity is required for maintenance of slow muscle phenotype and exercise-induced skeletal muscle adaptation.

In this study, we have used mice with targeted deletion of the Camk4 gene (Camk4<sup>−/−</sup>) to characterize different groups of muscle comprehensively and to assess muscle adaptation after long-term voluntary running for the expression of contractile and mitochondrial proteins. Our results show that CaMKIV activity is not required for the maintenance of the slow muscle phenotype or for exercise-induced adaptation in skeletal muscle. We have also obtained unambiguous results to indicate that CaMKIV protein is not expressed in murine skeletal muscle. Thus other protein kinases sharing substrates with constitutively active CaMKIV may function as endogenous mediators of activity-dependent changes in myofiber phenotype.

**MATERIALS AND METHODS**

**Animals.** Mice carrying a null mutation of the Camk4 gene were generated by using homologous recombination in embryonic stem cells as described previously (35). To characterize skeletal muscle phenotype in sedentary mice, soleus, plantaris, and white vastus lateralis muscles were harvested from the Camk4<sup>−/−</sup> mice and their wild-type littermates (C57BL/6J129/Sv; n = 5 for each group) after the mice were killed with an overdose of sodium pentobarbital (250 mg/kg body wt) delivered by intraperitoneal (ip) injection. The muscle samples were processed for Western immunoblot and indirect immunofluorescence analyses as described below. To determine the effects of long-term voluntary running on skeletal muscle, wild-type (C57BL/6J) and Camk4<sup>−/−</sup> mice (n = 6 for each group) were individually housed in cages (13 × 13 × 30 cm) equipped with running wheels (11 cm in diameter). Animals were maintained on a 12:12-h light-dark cycle, and wheel-running activity was monitored continuously with the Dataquest Acquisition and Analysis System (Data Sciences International, St. Paul, MN). After 4 wk of voluntary running, plantaris muscles were harvested from each mouse as described above for Western immunoblot analysis. To induce muscle contraction by nerve stimulation, a separate group of Camk4<sup>−/−</sup> mice and their wild-type littermates (n = 5 for each genotype) were used. Electrode implantation was performed as described previously in rats (38). Briefly, while the animals were under anesthesia (50 mg/kg sodium pentobarbital ip), their hindlimbs were shaved with an electric hair clipper and treated with iodine applied twice, followed by 70% ethanol applied twice. An incision of ~1 cm was made on the left lateral side of the thigh to expose the deep peroneal nerve. Two stainless steel electrodes were secured under the deep peroneal nerve, with two sutures used to fix each electrode. To accommodate the change in the size of the animal, the two electrodes were kept ~3 mm apart. Motor nerve stimulation was initiated within 30 min after surgery and lasted for 2 h, during which time the mouse was kept under anesthesia. The stimulation parameters were 0.25-ms duration and 10-Hz frequency as previously described (38), except that the amplitude was adjusted between 1 and 3 V to maintain maximal contractions and minimize possible damage. The contralateral tibialis anterior (TA) muscle was used as a control after performing a sham operation without stimulation. Immediately after motor nerve stimulation, both the stimulated and the contralateral control TA muscles were harvested and processed for total RNA preparation. All experimental protocols were approved by the Duke University Institutional Animal Care and Use Committee.

**Western immunoblot analysis.** Immediately after tissue procurement, skeletal muscles were homogenized in glass homogenizers in 0.3 ml of complete protein loading buffer containing 50 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 20 mM dithiothreitol, 127 mM 2-mercaptoethanol, and 0.01% bromophenol blue, supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). The muscle homogenates were transferred to microfuge tubes, heated for 5 min at 100°C, and centrifuged in a microfuge for 5 min at 13,000 rpm at room temperature. Protein concentration of each sample was determined by amido black protein assay (24), and 10 μg (for contractile proteins) or 40 μg (for other proteins) of total protein was resolved on SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and immunodetected by using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). The following antibodies were used for immunoblot analysis: PGC-1α polyclonal antibody (catalog no. SC-13067; Santa Cruz Biotechnology, Santa Cruz, CA), α-tubulin antibody (catalog no. 13-8000; Zymed Laboratories, South San Francisco, CA), myosin heavy chain (MHC) mouse monoclonal antibodies (catalog no. BF-F8 for MHC type I, catalog no. SC-71 for MHC type IIa, and catalog no. BF-F3 for MHC type IIb; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), myoglobin polyclonal antibody (catalog no. A0324; Dako, Carpinteria, CA), cytochrome c oxidase IV (COXIV) antibody (catalog no. A-21348; Molecular Probes, Eugene, OR), CaMKIIα (catalog no. SC-1542; Santa Cruz Biotechnology), CaMKIIβ antibody (catalog no. SC-1541; Santa Cruz Biotechnology), and CaMKI antibody (generous gift from A. R. Means, Dept. of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC). The intensities of the bands were quantified by using Scion Image software (Scion, Frederick, MD).

**Indirect immunofluorescence.** For fiber type composition determination, soleus muscles were harvested and frozen in isopentane cooled in liquid nitrogen. Frozen cross sections (5 μm) were immunostained using monoclonal antibodies against types I, IIA, and IIB. Briefly, muscle sections were fixed in 4% paraformaldehyde/ PBS for 10 min at 4°C and permeabilized with 3% Triton X-100/PBS for 10 min at 4°C. The sections were blocked in 5% normal goat serum (NGS)-PBS for 30 min at room temperature, followed by incubation with MHC type IIB antibody (BF-F3) diluted 1:100 in 5% NGS-PBS at 4°C.
overnight. The muscle sections were washed three times with PBS for 5 min each, followed by incubation with fluorescein-conjugated goat anti-mouse IgM secondary antibody (1:50 dilution) at room temperature for 30 min. The muscle sections were then washed three times with PBS and fixed in 4% paraformaldehyde for 2 min at 4°C and blocked with 5% NGS-PBS for 30 min. The sections were then sequentially stained as described above with MHC type I antibody (BA-F8, 1:100 dilution), followed by rhodamine red-X-conjugated

Fig. 1. Expression of contractile proteins and proteins involved in mitochondrial biogenesis in skeletal muscles of mice with targeted deletion of the Camk4 gene (Camk4<sup>−/−</sup>) and their wild-type littermates. A: Western immunoblot analysis of myosin heavy chain (MHC) types I, IIa, and IIb, myoglobin (Mb), peroxisome proliferator-activated receptor γ-coactivator 1α (PGC-1α), and cytochrome c oxidase IV (COXIV) proteins in soleus (SO), plantaris (PL), and white vastus lateralis (WV) skeletal muscles in Camk4<sup>−/−</sup> mice (KO) and the wild-type littermates (WT). α-Tubulin level was measured as a control for the loading and quality of the protein samples. B: quantification of the relative abundance of the proteins after normalization to the abundance of α-tubulin protein. (n = 5). **P < 0.01 vs. wild-type littermates.
Fig. 2. Camk4^-/- mice have a higher percentage of type I fibers in SO muscle. Indirect immunofluorescence staining of type I (rhodamine red-X, red), type IIa (cyanine Cy5, blue), type IIb (fluorescein, green), and other (no staining) myofibers in SO muscles in sedentary KO and WT. There are no type IIb fibers in these images, and an increased percentage of type I myofibers with a concurrent decrease in type IIa myofibers is evident in Camk4^-/- mice.

goose anti-mouse IgG and MHC type IIa antibody (SC-71, 1:100 dilution), followed by cyanine Cy5-conjugated goat anti-mouse IgG. Images were captured under an Olympus confocal microscope, and total myofibers of each type were counted to calculate fiber type composition for each muscle section. Myofiber diameter for types I and IIa (n > 300 for each section and each fiber type) was measured by using Scion Image software. The staining, photography, and image analysis were performed by a single individual who had no knowledge of the coding system.

Semiquantitative RT-PCR. Total RNA preparation and semiquantitative RT-PCR analysis were performed as described previously (37) to measure endogenous PGC-1alpha mRNA expression in TA muscle in response to increased contractile activity. PGC-1alpha mRNA data were normalized by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for each sample and expressed as the relative change compared with the contralateral control muscle. The PCR primers used were as follows: GAPDH forward primer, 5'-GTTGCAAGTGGAGATGTTGCC-3'; GAPDH reverse primer, 5'-GATGATGACCCGGTTGGCCTCA-3'; PGC-1alpha forward primer, 5'-AACCTTGCTAGCGGTCCTCA-3'; and PGC-1alpha reverse primer, 5'-TTTCTGTGGGTTTGGTGTGA-3'. Sequential denaturing (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 45 s) reactions were repeated 26 and 16 times for PGC-1alpha mRNA and GAPDH mRNA, respectively. The linearity of the PCR reactions was ensured by preliminary tests in which the most and least abundant samples were used with multiple reactions of different cycle numbers.

Statistics. Data are presented as means ± SE. For comparisons between the Camk4^-/- and wild-type mice and between the sedentary and trained mice, a two-tailed Student's t-test was used. For differences between the motor nerve-stimulated TA muscle and the contralateral control, a two-tailed, paired t-test was used. P < 0.05 was accepted as statistically significant.

RESULTS

Camk4^-/- mice have normal fiber type composition in fast-twitch muscles and an increased percentage of type I myofibers in slow-twitch soleus muscle. To comprehensively characterize skeletal muscle phenotype in sedentary mice, Western immunoblot analysis was performed to measure the expression of MHC proteins in three muscles of distinct fiber type composition: soleus (predominantly types I and IIa fibers), plantaris (predominantly types IIa and IIb fibers), and white vastus lateralis muscles (predominantly type IIb fibers). To our surprise, MHC type I protein concentration in soleus muscle was found to be ~100% higher (P < 0.01) in Camk4^-/- mice than that in the wild-type littermates (Fig. 1, A and B). Evidently, the change is specific to the soleus muscle, because no significant differences were observed in plantaris and white vastus lateralis muscles. To further confirm the finding in soleus muscle, we used indirect immunofluorescence to determine fiber type composition. Consistent with the Western immunoblot data, we observed a significantly higher percentage of type I fibers with a concurrent decrease in the percentage of type IIa fibers in the soleus muscles of Camk4^-/- mice (Fig. 2 and Table 1).

These morphological and biochemical differences in soleus muscle were associated with a 20% decrease in muscle mass (0.23 ± 0.01 mg/g body wt in Camk4^-/- mice vs. 0.29 ± 0.01 mg/g body wt in wild-type littermates, P < 0.05) (Table 1). No significant differences in muscle mass were observed in plantaris and TA muscles (predominantly type IIb fibers) between Camk4^-/- mice and their wild-type littermates (not shown).

The mean cross-sectional areas of the two predominant fiber types (types I and IIa) in soleus muscle were measured, and there was a trend toward decreased cross-sectional area for both types I and IIa fibers in Camk4^-/- mice relative to wild-type littermates, but the difference was not statistically significant (Table 1).

Table 1. Fiber type analysis of soleus muscle in Camk4^-/- mice and wild-type littermates

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 5)</th>
<th>KO (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle weight, mg/g body wt</td>
<td>0.29 ± 0.02</td>
<td>0.23 ± 0.01*</td>
</tr>
<tr>
<td>Fiber type composition, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>37.7 ± 1.5</td>
<td>57.5 ± 4.1*</td>
</tr>
<tr>
<td>Type IIa</td>
<td>51.9 ± 1.5</td>
<td>39.9 ± 3.9†</td>
</tr>
<tr>
<td>Type IIb</td>
<td>0.8 ± 0.4</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Other</td>
<td>9.6 ± 1.6</td>
<td>2.3 ± 0.3†</td>
</tr>
<tr>
<td>Cross-sectional area, μm²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>1,636 ± 99</td>
<td>1,542 ± 16</td>
</tr>
<tr>
<td>Type IIa</td>
<td>1,245 ± 141</td>
<td>1,178 ± 86</td>
</tr>
</tbody>
</table>

KO, Camk4^-/- mice; WT, wild-type littermate mice; Type I, myofibers stained positive for myosin heavy chain (MHC) type I with antibody BA-F8; Type IIa, myofibers stained positive for MHC type IIa with antibody SC-71; Type IIb, myofibers stained positive for MHC type IIb with antibody BF-F3; Other, myofibers with negative staining. Values are means ± SE. *P < 0.05 vs. WT. †P < 0.01 vs. WT.

AJP-Cell Physiol • VOL 287 • NOVEMBER 2004 • www.ajpcell.org
PGC-1α mRNA expression is induced in Camk4−/− mice in response to increased contractile activity. Several laboratories have reported that a single bout of contractile exercise is sufficient to induce a transient increase in PGC-1α mRNA expression in fast-twitch skeletal muscle in rats and humans (2, 18, 26). Investigators at our laboratory have observed similar changes in mice responding to a single bout of voluntary running or motor nerve stimulation (Pohnert SC, Akimoto T, Rosenberg PB, Williams RS, and Yan Z, unpublished observation). This induced expression of PGC-1α mRNA in skeletal muscle may play an important functional role in mediating the skeletal muscle adaptation. To determine whether CaMKIV activity is required for activity-dependent regulation of PGC-1α expression in skeletal muscle, we stimulated the TA muscle via the common peroneal nerve. Semiquantitative RT-PCR showed that short-term (2 h), low-frequency (10 Hz) muscle nerve stimulation resulted in a reproducible 150% (P < 0.01) and 100% increase (P < 0.01) in PGC-1α mRNA compared with the contralateral control TA muscle in the wild-type littermates and the Camk4−/− mice, respectively (Fig. 3, A and B).

Camk4−/− mice undergo normal fiber type switching and enhanced mitochondrial biogenesis in plantaris muscle after long-term voluntary running. To determine whether CaMKIV activity is required for skeletal muscle adaptation in response to endurance exercise, Camk4−/− mice were subjected to long-term voluntary running (4 wk), and myoglobin, PGC-1α, COXIV, and MHC proteins were measured in the active plantaris muscles. Camk4−/− mice ran an average of 3.9 ± 0.7 km/day at the beginning of the training, and the running distance increased to 5.7 ± 0.7 km/day after 28 days of voluntary running (Fig. 4A). The average running distance of the wild-type C57BL/6J mice was about twice that of the Camk4−/− mice before and after the long-term voluntary running (8.3 ± 1.1 km/day in sedentary mice and 10.7 ± 0.9 km/day after training). C57BL/6J mice are known to run the longest daily distance among different mouse strains on the voluntary running wheel (1) and were used as a wild-type control in this study for comparison. Consistent increases in myoglobin, PGC-1α, COXIV, and MHC type IIa protein expression similar to that observed in the C57BL/6J wild-type mice were noted in the plantaris muscle of Camk4−/− mice (Fig. 4, B and C).

CamKIV protein is not detectable in murine skeletal muscle.

To determine without ambiguity whether CaMKIV protein is expressed in murine skeletal muscles, proteins in the total cell lysates from the brain and soleus muscle were resolved using SDS-PAGE and immunoblotted with an anti-CaMKIV antibody (catalog no. 610275; BD Transduction Laboratories) with or without preincubation with recombinant CaMKIV protein. Consistent with the previous report (40), this antibody recognized CaMKIV protein as a dominant band at a molecular mass of ~61 kDa only in the brains of wild-type mice and not in the brains of Camk4−/− mice (Fig. 5). Interestingly, a protein with apparent molecular mass similar to that of CaMKIV was detected in the soleus muscles of both the wild-type and Camk4−/− mice. However, when the anti-CaMKIV antibody was preincubated with recombinant glutathione S-transferase-CaMKIV protein to titrate the active antibody, the ability of this antibody to recognize CaMKIV in the brain was completely abolished, while the recognition of the 61-kDa protein in the soleus muscle on the same membrane was not affected. We conclude that the antibody mixture detects a protein distinct from CaMKIV in skeletal muscle.

To rule out possible compensation by enhanced expression of other CaMK, we also performed Western blot analysis for CaMK1, CaMKIIγ, and CaMKIIδ in the soleus muscles of wild-type and Camk4−/− mice. There were no significant differences in the expression of these CaMK proteins (Fig. 6), confirming that the normal muscle phenotype in fast-twitch muscles in Camk4−/− mice is not due to compensatory mechanisms involving these CaMK proteins in skeletal muscle as a result of genetic disruption of the Camk4 gene.

**DISCUSSION**

The mechanisms of adult skeletal muscle adaptation have been the subject of investigation for more than four decades, and compelling evidence supports an important role for neuromuscular activity, particularly the motor neuron firing pattern, in the determination and maintenance of muscle mitochondrial biogenesis and fiber type composition (17, 27, 30). Accumulating evidence supports the view that Ca2+ signaling has a pivotal function in linking different patterns of motor

---

**Figure 3.** Contractile activity induces PGC-1α mRNA expression in tibialis anterior (TA) muscles of Camk4−/− mice and WT littermates. A: agarose gel image of semiquantitative RT-PCR analysis of PGC-1α mRNA in the stimulated (Stim) and contralateral control (Con) TA muscles. Samples from the same mouse are underlined. B: quantification of the relative abundance of PGC-1α mRNA in the stimulated and contralateral control TA muscles after normalization to the abundance of glyceraldehyde-3-phosphate dehydrogenase mRNA. **P < 0.01 compared with contralateral control muscle (n = 6).**
Fig. 4. Voluntary running induces skeletal muscle adaptation in KO and WT mice. 

A: average daily running distance before and after 4 wk of training increases in KO mice (C57BL/6J/129/Sv) and C57BL/6J WT mice (*n = 5*). +++P < 0.001 compared with WT mice.

B: Western immunoblot analysis of MHC I, IIa, and IIb, Mb, PGC-1α, and COXIV proteins in PL muscle in sedentary (S) and trained (T) KO mice and their WT littermates. SO muscle lysate was loaded as a control for MHC I protein expression. α-Tubulin level was measured for the loading and quality of the protein samples. 

C: quantification of relative abundance of the proteins after normalization to the abundance of α-tubulin protein (*n = 5*). *P < 0.05 vs. sedentary mice of the same genotype. **P < 0.01 vs. sedentary mice of the same genotype.
Camk4−/− mice showed an increased percentage of type I fibers and a concurrent decrease in type Ila fibers and muscle mass in soleus muscles. This is an unexpected finding that argues against our initial hypothesis. The decrease in muscle mass is likely due to a decrease in muscle fiber size, because we noticed a trend of decreased cross-sectional area of both types I and Ila fibers in the soleus muscles of Camk4−/− mice. The subtle, but not statistically significant, difference (~5%) in the mean cross-sectional area between the Camk4−/− mice and their wild-type littermates appears to be consistent with a volume reduction of 20%. It is known that CamKIV protein is highly expressed in developing spinal cord, including both dorsal root and sympathetic ganglia (9, 28), and plays a functional role (8, 21) in the central and peripheral nervous systems. The changes in fiber type composition and muscle mass in the soleus muscles of Camk4−/− mice may result from subtle neuronal defects that alter neural input to certain skeletal muscles in the absence of CamKIV activity. Further experimentation is required to address the functional role of this potential function of CamKIV in motor nerve.

The role for CamKIV within skeletal muscles in signal transduction arising from changes in neuromuscular activity was rigorously tested by subjecting Camk4−/− mice to 4 wk of voluntary running followed by a comprehensive phenotypic

nerve activity to distinctive programs of gene expression that establish phenotypic diversity among skeletal myofibers (14).

Gain-of-function and loss-of-function studies have suggested that stimulation of the calcineurin pathway, leading to activated NF-AT and MEF2 transcription factors, plays an essential role in slow muscle fiber specialization (5, 11, 16). More recent findings have suggested the functional role for a parallel CaMK pathway in supporting slow muscle gene expression (33). For example, investigators at our laboratory have demonstrated that overexpression of a constitutively active form of CaMKIV in the skeletal muscles of transgenic mice triggers significant mitochondrial biogenesis and fast-to-slow fiber type transformation, along with enhanced expression of PGC-1α (32). Other groups also have observed activation of PGC-1α gene expression and promoter activity by ectopic expression of this active form of CaMKIV (7, 40). In addition, Ca2+ signaling induces PGC-1α protein and mRNA expression in cultured myotubes in a CaMK activity-dependent manner (12). Furthermore, enhanced mitochondrial biogenesis in skeletal muscle in response to energy starvation has been suggested to be associated with increased CamKIV protein expression in a AMPK-dependent manner (40). An important remaining question is whether CaMKIV is part of the physiological mechanism by which skeletal muscle undergoes adaptation in response to stimuli such as endurance exercise in vivo. Mice with deletion of the Camk4 gene (36) provided an opportunity to address this important question in a loss-of-function manner.

If endogenous CaMKIV participates in signaling mechanisms that establish and maintain the slow muscle fiber type, one would expect targeted mutation of the Camk4 gene to result in a decreased percentage of types I and Ila myofibers and a concurrent increase in the percentage of type Iib fibers, as well as decreased expression of mitochondrial proteins, in skeletal muscle. The results of this study show clearly that the maintenance of slow muscle fibers and basal level mitochondrial biogenesis are not dependent on CaMKIV activity in skeletal muscle. Thus we conclude that CaMKIV activity is not required for the maintenance of slow muscle fiber type and mitochondrial biogenesis in skeletal muscle.
analysis. A comparison of adaptability was made with age-matched C57BL/6J wild-type mice. Although Camk4−/− mice (in C57BL/6J129/Sv background) demonstrated shorter daily running distances, we have biochemical evidence that Camk4−/− mice undergo skeletal muscle adaptation similar to that of wild-type C57BL/6J mice, including fiber type switching in response to increased contractile activity. Furthermore, expression of PGC-1α mRNA and protein in Camk4−/− mice was induced by increased contractile activity in a manner similar to that of the wild-type littermates. These findings indicate that CaMKIV is not required for exercise-induced mitochondrial biogenesis and Iib-to-Ila fiber type switching in skeletal muscle. Therefore, CaMKIV is not a physically relevant component of the signaling circuitry.

The noticeable difference in running distance between the Camk4−/− mice (in C57BL/6J129/Sv background) and wild-type mice (in C57BL/6J background) (Fig. 4A) is likely due to the difference in the genetic background as reported previously in a comprehensive comparison of various strains of mice using the voluntary running model (1). The above-mentioned potential neural defect may also contribute to the difference in running distance. Nevertheless, despite the fact that the average daily running distance is shorter in the Camk4−/− mice, these mice demonstrated adaptability similar to that of the wild-type mice on the basis of contractile and mitochondrial protein measurements.

The findings in sedentary and trained Camk4−/− mice indicate that CaMKIV does not have a functional role in establishing the slow fiber genetic program in skeletal muscle. Several interpretations may help to reconcile the present findings with previously reported observations. First, Ca2+ signals arising as a function of changes in contractile work may be transduced through a different isoform of CaMK, such as CaMKI or CaMKII, or by other protein kinases that share substrates with the constitutively active form of CaMKIV. Previous findings that muscle contraction activates CaMKII activity are consistent with this notion (6, 22). We are actively studying the functional role of the other candidates in this regard. Second, a compensatory mechanism may induce the expression of related protein kinases that indirectly mitigate the effects of CaMKIV. Our finding that expression of CaMKI and some CaMKII isoforms was not altered in Camk4−/− mice suggests that compensation in the expression of CaMK isoforms is not likely the mechanism. Finally, it is possible that activation of yet to be determined CaMK-dependent pathways might be sufficient, but not required, for induction of skeletal muscle adaptations to increased work activity. Future research should address the functional role of other CaMK in skeletal muscle adaptation in response to increased contractile activity.

CaMKIV protein was reported to be undetectable in skeletal muscle in some previous studies, but the analysis was not exhaustive (22, 32). Other reports described that CaMKIV protein is expressed in skeletal muscle and can be further induced by energy deprivation to mediate enhanced mitochondrial biogenesis in skeletal muscle (40). To resolve this issue and obtain accurate information of CaMKIV protein expression in skeletal muscle, we performed a comprehensive analysis. We now present additional evidence that no detectable CaMKIV is expressed in skeletal muscle. A direct comparison of brain tissue lysates between Camk4−/− mice and their wild-type littermates allowed us to confirm the band on im

munoblots representing endogenous CaMKIV protein in the wild-type mice with great certainty, because the CaMKIV protein band was not detectable in the brain extract of Camk4−/− mice. Preincubation with recombinant CaMKIV protein to titrate the antibody abolished the detection of this band in the brain extract of wild-type mice, further proving its identity. A protein band detected at approximately the molecular size predicted for CaMKIV was evident in the soleus muscles of both Camk4−/− and their wild-type littermates, suggesting that this is not CaMKIV, because targeted mutation did not affect its expression. Furthermore, detection of this protein was unaffected by preincubation with recombinant CaMKIV protein, providing strong evidence that this protein is not CaMKIV but a cross-reacting protein that is not a product of the Camk4 gene. The expression of this cross-reacting protein is markedly induced by motor nerve stimulation (data not shown), consistent with the previous finding that its expression is sensitive to changes in energy metabolism (40).

The confirmation that CaMKIV is not expressed in skeletal muscle is valuable to understanding of our findings that Camk4−/− mice are normal in muscle fiber type composition and with regard to activity-dependent changes in myofiber specialization. These findings suggest that future research should focus on the potential functional roles of CaMKI, CaMKII, CaMKK, or other protein kinases that act on substrates shared with the constitutively active form of CaMKIV as mediators of skeletal muscle plasticity.

ACKNOWLEDGMENTS

We thank Drs. A. R. Means and J. M. Colomer Font for careful review of the manuscript. We appreciate the excellent technical support of Mei Zhang, C. Ireland, and C. Gumbs.

GRANTS

This work was supported by American Heart Association Grant 0130261N (to Z. Yan) and National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-40849 (to R. S. Williams). T. Akimoto is a recipient of a Grant-in-Aid for Overseas Research Scholars from the Ministry of Education, Science, and Culture of Japan. The Camk4−/− mice were generated previously with the support of National Institute of Child Health and Human Development Grant HD-07503 (to A. R. Means).

REFERENCES


