Skeletal muscle calcineurin: influence of phenotype adaptation and atrophy

ESPEN E. SPANGENBURG,1 JAY H. WILLIAMS,1 ROLAND R. ROY,2 AND ROBERT J. TALMADGE 1
1Muscle Function Laboratory, Department of Human Nutrition, Foods, and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061; and 2Brain Research Institute, University of California Los Angeles, Los Angeles, California 90095

Spangenburg, Espen E., Jay H. Williams, Roland R. Roy, and Robert J. Talmadge. Skeletal muscle calcineurin: influence of phenotype adaptation and atrophy. Am J Physiol Regulatory Integrative Comp Physiol 280: R1256–R1260, 2001.—Calcineurin (CaN) has been implicated as a signaling molecule that can transduce physiological stimuli (e.g., contractile activity) into molecular signals that initiate slow-fiber phenotypic gene expression and muscle growth. To determine the influence of muscle phenotype and atrophy on CaN levels in muscle, the levels of soluble CaN in rat muscles of varying phenotype, as assessed by myosin heavy chain (MHC)-isoform proportions, were determined by Western blotting. CaN levels were significantly greater in the plantaris muscle containing predominantly fast (IIx and IIb) MHC isoforms, compared with the soleus (predominantly type I MHC) or vastus intermedius (VI, contains all 4 adult MHC isoforms). Three months after a complete spinal cord transection (ST), the CaN levels in the VI muscle were significantly reduced, despite a significant increase in fast MHC isoforms. Surprisingly, the levels of CaN in the VI were highly correlated with muscle mass but not MHC isoform proportions in ST and control rats. These data demonstrate that CaN levels in skeletal muscle are highly correlated to muscle mass and that the normal relationship with phenotype is lost after ST.

calcium; calmodulin; myosin heavy chain; nuclear factor of activated T cells

SKELETAL MUSCLE FIBER TYPE DIVERSITY (i.e., muscle fiber phenotype) is, in part, distinguished by various muscle protein isoforms [e.g., myosin heavy chain (MHC), myosin light chain, sarco(endo)plasmic reticulum calcium ATPase (SERCA), and lactate dehydrogenase isoforms] that influence contractile physiology and metabolic properties (20). Alterations in the level of expression of these protein isoforms, as well as other proteins, can be induced by physiological stimuli such as altered levels of muscle activation and load bearing (11, 14, 18). At present, the intracellular pathways that transduce physiological stimuli into molecular signals that alter phenotypic gene expression are largely unknown.

Recent reports suggest that calcineurin (CaN), a calcium-regulated 2B protein phosphatase, may be responsible for the electrical activity-based regulation of fiber phenotypic transformations (4, 25). It is hypothesized that CaN acts as a sensor of contractile activity by sensing Ca$^{2+}$ signals. The signaling mechanism is initiated via sustained elevations of free myoplasmic Ca$^{2+}$, allowing Ca$^{2+}$ to bind to calmodulin, which, in turn, activates the phosphatase activity of CaN via the regulatory subunit. The activation of CaN then initiates the translocation of the nuclear factor of activated T cells (NF-AT) and CaN from the cytoplasm to the nucleus via dephosphorylation of NF-AT. Once in the nucleus, NF-AT is hypothesized to act as a transcriptional activator of slow muscle specific genes (4). The idea that CaN may play a role in regulating phenotypic gene expression is appealing because contractile activity, which is known to affect fiber phenotype, is ultimately controlled by free intracellular calcium transients.

Supporting this idea, the in vivo administration of cyclosporin A (CsA), a potent inhibitor of CaN, has been shown to prevent fast-to-slow MHC-isoform transitions under conditions of functional overload (5). CsA also inhibits the expression of type I MHC in electrically stimulated myotube cultures (8). Similarly, CsA administration results in elevated proportions of fast muscle fibers in the soleus muscle (a highly active postural muscle) of normal rats (4). Although CsA may also inhibit other enzymatic systems, the data are consistent with the proposed role of CaN as a signaling molecule involved in fiber phenotype regulation. How...
ever, the relationship between CaN and MHC-isoform composition was previously unknown.

Therefore, the purpose of this investigation was to determine whether CaN levels were correlated with MHC-isoform content under control conditions and conditions known to induce phenotypic adaptation. The data indicate that in control muscles, CaN protein expression was highly correlated with MHC-isoform composition. Surprisingly, the correlation was such that muscles with fast MHC isoforms contained higher amounts of CaN than muscles with slow MHC isoforms. Curiously, the relationship between CaN levels and MHC-isoform composition was lost after spinal cord transection, which caused an increase in fast MHC isoforms and a reduction in CaN.

MATERIALS AND METHODS

Animals. For all experiments, muscles were removed from anesthetized female Sprague-Dawley (~200 g, n = 6–8 per group) rats. The plantaris, vastus intermedius (VI), and soleus muscles were quickly removed, trimmed of connective tissue, weighed, frozen in melting isopentane cooled by liquid nitrogen, and stored at −80°C for subsequent analysis. For the spinal cord transection (ST) studies, eight female Sprague-Dawley rats weighing ~150 g were subjected to a complete transection of the spinal cord at a midthoracic level, as described in detail previously (21, 22), and maintained for 3 mo. The VI was chosen for study after ST because it shows a high degree of atrophy in rats (R. J. Talmadge, R. R. Roy, and V. R. Edgerton, unpublished observations) and cats (24) and is normally comprised of a mixture of all four adult MHC isoforms.

Muscle preparation. The frozen muscles were minced in 2 ml of buffer containing (in mM) 250 sucrose, 5 EDTA, 100 KCl, and 20 HEPES (pH 7.0). Subsequently, the muscles were homogenized by a motorized homogenizer. The sample was then centrifuged at 1,000g for 10 min at 5°C. The supernatant (representing the cytosolic fraction) was used for CaN measurements, whereas the pellet was resuspended in 2 ml of buffer and used for MHC-isoform analyses. Total protein of each fraction was determined according to Bradford (2). The samples for MHC-isoform and CaN analyses were boiled for 2 min in sample buffer at final concentrations of 0.125 and 2.0 mg/ml, respectively.

Western blotting for CaN. Cytosolic proteins were separated by SDS-PAGE on 7.5% total acrylamide gels, as previously described by Laemmli (7). Forty micrograms of total protein were loaded per lane. The proteins were blotted onto nitrocellulose membranes (23). The membranes were then blocked in 5% nonfat dry milk dissolved in PBS for 1 h and incubated overnight with a monoclonal antibody raised against the α-subunit of CaN (CN-A1, Sigma) diluted 1:10,000 in PBS. An anti-mouse IgG conjugated to alkaline phosphatase was used as the secondary antibody. The membranes were then incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate for the visualization of alkaline phosphatase activity and subsequently scanned, photographed, and analyzed with an Alpha Innotech IS-2000 video densitometric system.

Statistics. Values are expressed as means ± SE. Multi-group (i.e., muscle) comparisons were performed by one-way ANOVA followed by Student-Neuman-Keuls post-ANOVA tests. Student’s t-tests were used for comparisons between treatment groups (ST vs. control) for the VI muscle. For all comparisons, the alpha level was set at P ≤ 0.05. Correlational analyses were performed using the SigmaStat statistical package.

RESULTS

Soluble CaN (α-subunit) levels varied significantly among the three muscles studied (Fig. 1). To determine whether muscle phenotype was related to CaN protein levels, the relative MHC-isoform profiles of the muscles were determined (Fig. 2). The soleus contained no detectable IIx or IIb MHCs, whereas the VI and the plantaris contained 27% and 79% MHC IIx and IIb combined, respectively. Type I MHC percentage was 88% in the soleus, 48% in the VI, and 6% in the plantaris. Correlations between MHC-isoform percent-
age and CaN expression among the three muscles revealed a significant negative correlation with MHC I percentage ($r^2 = 0.59, P \leq 0.05$) and a significant positive correlation ($r^2 = 0.56, P \leq 0.05$) with the percentage of MHCs IIx and IIb combined (Fig. 3).

Three months after ST, the VI showed a shift toward faster MHC isoforms (Fig. 4) and a significant reduction (by $\sim 50\%$) in the Western blot signal for CaN (2.26 ± 0.22 in control vs. 1.10 ± 0.15 in ST rats, $P \leq 0.05$). The MHC isoform content and CaN levels of the individual VI muscles were poorly correlated in the ST and control rats (Table 1).

The VI showed significant muscle atrophy after ST, such that absolute mass was significantly reduced by $\sim 40\%$ compared with control values (206 ± 13 mg in control vs. 122 ± 13 mg in ST rats, $P \leq 0.05$), and relative mass (corrected for body mass) showed a $\sim 35\%$ decrease (0.779 ± 0.044 mg/g body mass in control vs. 0.504 ± 0.055 mg/g body mass in ST rats, $P \leq 0.05$). The CaN levels and muscle mass of the VI were positively correlated ($r^2 = 0.61, P \leq 0.05$) for control and ST rats (Fig. 5).

**DISCUSSION**

It has been suggested that CaN may be involved in regulating phenotypic protein expression by dephosphorylating cytosolic NF-AT, resulting in NF-AT translocation into the nucleus (4–6) and enhanced myocyte enhancer factor 2A (MEF2A) signaling (25). The NF-AT and MEF2A present in the nucleus would then activate slow muscle phenotypic genes (4–6, 25). Chin et al. (4) suggested that slow postural muscles, which maintain higher levels of resting myoplasmic Ca$^{2+}$, would sufficiently activate NF-AT translocation via a CaN-dependent process and maintain slow phenotypic protein expression through NF-AT activation of slow muscle-specific genes. It was also suggested that, due to the low resting levels of myoplasmic Ca$^{2+}$ and short durations of Ca$^{2+}$ amplitudes observed in
fast fibers during activation, fast fibers would maintain lower levels of CaN activity resulting in low levels of nuclear NF-AT and inactivation of slow muscle-specific genes (4). Also, transgenic mice expressing a constitutively active (and not calcium regulated) form of CaN have increased numbers of slow fibers (as determined by myofibrillar ATPase), increased expression of myoglobin, troponin I slow, and mitochondrial creatine kinase, and decreased expression of parvalbumin and skeletal muscle creatine kinase in hindlimb muscle (10). Furthermore, inactivation of CaN phosphatase activity via CsA administration results in elevations in fast fibers in the rat soleus muscle (4) and prevents the overload-induced transformation of fibers from a fast to a slow phenotype (5). These data support a role for calcium-calmodulin-activated CaN in modulating fast-to-slow phenotypic transformations.

CaN appears to be required for skeletal muscle hypertrophy, whether induced by overload or growth factors (5, 9, 15). This indicates that CaN may mediate one of the multiple mechanisms involved in muscle growth regulation. Collectively, these data suggest that CaN plays important roles both in growth and in phenotype regulation of skeletal muscle.

Our data suggest that under normal conditions, CaN is distributed in a fiber phenotype-specific fashion. Specifically, muscles containing higher proportions of fast phenotypic proteins, such as the type IIX and IIb MHC isoforms, contain higher levels of CaN. This seems to be in contrast with the hypothesized role of CaN in maintaining and initiating slow muscle fiber characteristics. For example, if elevated contractile activity induces elevations in nuclear NF-AT and subsequent slow muscle phenotypic gene transcription, then one might expect that slow fibers would have higher CaN protein (and CaN phosphatase activity) than fast fibers. Thus there is an apparent discrepancy between the proposed role for CaN in skeletal muscles and its fiber-type distribution.

One potential explanation for the observation that CaN levels are greater in fast than in slow muscles may relate to muscle fiber size. Multiple studies (12–14) have shown that fibers containing type IIB or IIX MHC isoforms have significantly larger cross-sectional areas than fibers containing type I or IIa MHC. Thus it is possible that the greater levels of CaN in IIb and IIX fibers are more closely related to fiber size than fiber phenotype. Elevated levels of CaN may be required for muscle fibers to achieve a particular size. Because CaN is known to play a role in the regulation of cell size both in skeletal (5, 9, 15) and in cardiac (16) muscle, this explanation is plausible. Furthermore, we have found that, in mice expressing a transgene consisting of the MCK promoter and a constitutively active form of CaN, slow muscles are significantly hypertrophied compared with wild-type litter mate controls (R. J. Talmadge, S. Weber, J. S. Otis, F. J. Naya, and E. N. Olson, unpublished observations).

Our data demonstrating a reduction in CaN levels in the atrophied rat VI after ST are in agreement with the idea that CaN plays a role in regulating muscle mass. After ST, substantial levels and activity of CaN may not be required to support the decreased muscle mass. In fact, it is possible that reductions in muscle activity and load bearing brought about by ST may use CaN as an intracellular signaling mechanism to initiate muscle atrophy.

Another possible explanation for the greater levels of CaN in fast fiber types might relate to the observation that the fast IIB MHC isoform appears to be positively regulated in a CaN-dependent fashion (17). This idea is in direct contrast to the initial hypothesized role of CaN as a contractile activity sensor and positive signaling molecule for slow phenotypic gene expression (4). Furthermore, Calvo et al. (3) demonstrated that disruption of the NF-AT response element (i.e., the region of DNA that binds NF-AT) in the upstream regulatory element of slow troponin I (i.e., SURE) did not disrupt slow fiber-specific expression of slow troponin I. Thus the CaN and NF-AT pathway may not be important for fiber type-specific expression of all muscle fiber type-specific genes. This is supported by the observation that the normal phenotypic relationship between CaN levels and MHC-isoform protein expression was lost after ST.

A third explanation is that fast (type IIX and IIb) fibers may require higher levels of CaN to maintain an

### Table 1. Linear relationships between the levels of CaN in a muscle and various measures of phenotype for the VI muscle of ST and control animals

<table>
<thead>
<tr>
<th>Phenotype Measure (MHC-Isomorph Proportion)</th>
<th>Linear Regression Coefficient of Determination (r²)</th>
<th>Linear Regression Significance Level (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC I</td>
<td>0.194</td>
<td>0.088</td>
</tr>
<tr>
<td>MHC IIa</td>
<td>0.047</td>
<td>0.420</td>
</tr>
<tr>
<td>MHC IIX</td>
<td>0.250</td>
<td>0.049</td>
</tr>
<tr>
<td>MHC IIb</td>
<td>0.016</td>
<td>0.639</td>
</tr>
<tr>
<td>MHCs IIX + IIb</td>
<td>0.190</td>
<td>0.091</td>
</tr>
</tbody>
</table>

CaN, calcineurin; VI, vastus intermedius; MHC, myosin heavy chain; ST, spinal cord transection.

Fig. 5. Relationships between VI muscle mass and normalized CaN levels in control (■) and ST (□) rats. CaN levels were positively and linearly correlated with muscle mass (r² = 0.61, P ≤ 0.05).
appropriate level of sensitivity of gene expression to Ca\(^{2+}\) fluctuations. Because Ca\(^{2+}\) transients are less frequent in fast than in slow muscle fibers, fast fibers may require higher levels of CaN to increase the probability of a Ca\(^{2+}\)-calmodulin activation of CaN to induce fast-to-slow muscle fiber transformations should the muscle be activated appropriately. In contrast, slow fibers may require less CaN, because they are activated more frequently and encounter more frequent Ca\(^{2+}\)-release events. Thus these fibers may experience sufficient activation of CaN to insure slow fiber phenotypic gene expression, despite low levels of soluble CaN.

Finally, it is possible that in fast muscles, the majority of CaN was in the cytosolic fraction, whereas in slow muscles, a majority of CaN may be in the nucleus due to Ca\(^{2+}\)-(activity)-induced translocation of the NF-AT/CaN complex into the nucleus. This would be consistent with observations made by Bigard et al. (1) showing that nuclear CaN appears to be greater in slow fiber nuclei.

The data presented here clearly demonstrate that soluble CaN is normally distributed in a muscle phenotype- and muscle mass-specific fashion, suggesting that CaN plays some role in regulating the phenotypic properties (including fiber size and contractile properties) of muscle fibers. Whether this role is related to the regulation of muscle fiber size, muscle protein isoform expression, or both remains to be determined. However, our observation that the phenotypic, but not the muscle mass, relationship to CaN levels was lost after ST suggests that CaN may play a more important role in regulating muscle mass than phenotype. Future studies should be aimed at determining the factors that regulate the expression of CaN in a phenotype-specific manner and the precise role played by CaN in defined fiber types.

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