Calcineurin differentially regulates maintenance and growth of phenotypically distinct muscles

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Received 10 October 2001; accepted in final form 3 December 2001

Calcineurin differentially regulates maintenance and growth of phenotypically distinct muscles. Am J Physiol Cell Physiol 282: C984–C992, 2002. First published December 5, 2001; 10.1152/ajpcell.00483.2001.—Adequate muscle mass is critical for human health. The molecular pathways regulating maintenance and growth of adult skeletal muscle are little understood. Calcineurin (CN) is implicated as a key signaling molecule in hypertrophy. Whether CN is involved in all forms of muscle growth or in different muscles is unknown. Here, we examine the role of CN in regulating maintenance of muscle size and growth of atrophied muscle in the soleus (slow) and plantaris (fast). The CN inhibitor cyclosporin A (CsA) differentially affects muscle growth and maintenance depending on muscle phenotype. The plantaris is more severely affected by CsA than the soleus in both growth conditions. One-week vs. 2-wk CsA treatment suggests that both CN-dependent and CN-independent growth occur in the atrophied soleus, whereas plantaris growth appears to be totally CN dependent. Our results suggest that CN regulates multiple types of muscle growth, depending on muscle phenotype and stage of myofiber growth. Differential expression of components of the CN pathway occurs and may contribute to the differences between muscles.

muscle precursor cells; myonuclear number; myofiber growth; cyclophilin

SKELETAL MUSCLE COMPRISSES ~50–60% of total body mass and is one of the major tissues involved in regulating metabolism, locomotion, and strength. A correlation exists between total muscle mass and morbidity, such that a severe reduction in muscle mass, induced through a variety of different conditions, leads to increased disability, morbidity, and mortality. Understanding the mechanisms that regulate growth and maintenance of skeletal muscle is therefore critical for developing countermeasures to prevent muscle loss and ensure the health and quality of life of an individual.

Muscle growth occurs in adults in several situations. These include the increase in muscle size that occurs when normal muscle is subjected to increased loading conditions (hypertrophy) or upon cessation of an atrophic stimulus. Maintenance of normal muscle mass also requires a subtle balance between muscle growth and processes leading to muscle degradation. Whether the molecular and cellular mechanisms regulating muscle size in these different situations are similar is largely unknown. Another important question is whether growth and maintenance of all muscles are controlled by the same mechanisms.

We (25) and others (17) have demonstrated that differences exist in the cellular mechanisms regulating muscle growth. Specifically, the relative contribution of muscle precursor cells (MPCs) differed, depending on the type of growth and muscle type. MPCs were required for growth of the soleus (slow-twitch) but not the plantaris (fast-twitch) after different atrophic stimuli (17, 25). The study by Rosenblatt and Parry (31) showed that elimination of MPC from the extensor digitorum longus by gamma irradiation completely prevented overload-induced hypertrophy, whereas similar elimination of MPC from the same muscle only attenuated insulin-like growth factor I (IGF-I)-induced hypertrophy (4). Taken together, these studies demonstrate that different muscles employ different cellular mechanisms during growth. Moreover, in our previous study, we demonstrated that an MPC-dependent pathway contributes to growth of the soleus after atrophy only at a later stage during growth, suggesting that muscle growth occurs in stages, each of which may be regulated by different mechanisms (25).

The molecular mechanisms responsible for muscle growth are less well understood; however, calcineurin (CN) has emerged as a potential key signaling molecule in both skeletal (15, 16) and cardiac (26, 27) hypertrophy. CN is a Ca2+/calmodulin-dependent serine/threonine phosphatase that is activated in response to sustained increases in intracellular Ca2+ (14). The majority of these studies employed the immunosuppressive drug cyclosporin A (CsA) to inhibit CN activity; however, targeted expression of CAIN, a specific protein inhibitor of CN, in overloaded hearts attenuated cardiac hypertrophy. CN is also implicated...
in maintenance of muscle homeostasis. A recent report describes a correlation between CN levels and muscle mass (34), and specific muscles react to long-term treatment with CsA by changes in contractile and metabolic components (6, 7, 11). The downstream molecular and cellular targets by which CN exerts its effects on muscle growth are not well characterized.

The purpose of this study was threefold. The first goal was to determine the effects of CsA on the growth of atrophied muscle. The second was to determine whether CsA differentially affects growth and maintenance of phenotypically distinct muscles such as the slow-twitch soleus and the fast-twitch plantaris. Last, we sought to determine whether CsA affects early and late stages of muscle growth equally. Our results suggest that CN-dependent and CN-independent pathways contribute to muscle growth, depending both on muscle phenotype and the particular stage of growth. Molecular components involved in the CN signaling pathway are expressed differentially between the soleus and plantaris and may contribute to the differences observed in response to CsA treatment.

MATERIALS AND METHODS

Animals. Female Balb/c mice (9–11 wk) were purchased from Charles River (Wilmington, MA). All mice were housed under a 12:12-h light-dark cycle, and food and water were provided ad libitum. All procedures were approved by Emory University's Institutional Animal Care and Use Committee.

Mice were randomly assigned to one of three groups: 1) hindlimb suspension (HS), 2) HS and recovery, or 3) control. In group 1, mice were hindlimb suspended for 2 wk using a tail-suspension device as described previously (25). In group 2, HS mice after 2 wk were briefly anesthetized with xylazine (10 mg/kg) and ketamine (65 mg/kg), and the tail harness was removed. The length of the recovery period varied depending on the particular experiment. In groups 1 and 2, the mice were monitored twice daily throughout the period of HS. In group 3, mice maintained normal cage activity throughout each experimental procedure.

In some experiments, one hindlimb was gamma irradiated as previously described (25). Anesthetized mice were shielded so that only one of the hindlimbs was exposed to a cobalt-60 source. A dose of 2,500 rads was applied over 15 min, and the dose was verified using a picoameter ionization probe (LND, Oceanside, NY). This dose of irradiation has been previously shown to inhibit MPC proliferation (25). HS was started 2 days after the irradiation procedure.

Some mice in groups 2 and 3 were injected intraperitoneally once daily with vehicle or CsA (12, 25, or 50 mg/kg) for 1 or 2 wk, depending on the particular experiment. Injections of vehicle consisted of 7.8% cremophor EL (Sigma, St. Louis, MO) and 4.0% ethanol in sterile PBS. CsA was obtained from Bedford Laboratories (Bedford, OH). One day before starting injections, mice were switched to antibiotic drinking water as described previously (29). Administration of CsA at all doses did not appear to affect the health or normal cage activity of these mice.

Collection of soleus and plantaris muscles. Mice were euthanized by CO2 inhalation, and both soleus muscles and plantaris muscles were collected using standardized dissection methods and cleaned of excess fat and connective tissue. In some experiments, one muscle was snap frozen in liquid nitrogen for biochemical assays, whereas the contralateral muscle was embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and frozen in isopentane cooled in liquid nitrogen. In experiments where one hindlimb was gamma irradiated, both muscles were similarly embedded and frozen. These muscles were subsequently used to determine both cross-sectional area and myonuclear number of myofibers. For histological assays, 8-μm serial sections were collected from the belly region of the muscle, air-dried, and subsequently used for the immunohistochemical assays described below.

Myofiber cross-sectional area and myonuclear number analyses. A mouse monoclonal antibody against dystrophin (MANDYS8) was purchased from Sigma Biosciences (St. Louis, MO). Texas Red-conjugated goat anti-mouse IgG was purchased from Cappel (Durham, NC). Vectashield mounting media was purchased from Vector Labs (Burlingame, CA). All analyses and photography were performed on an Axiosplan microscope (Zeiss, Germany) equipped with a video camera and Scion Image and Adobe Photoshop software. The cross-sectional area of myofibers was determined by capturing nonoverlapping images in the center of sections from the belly of each muscle and analyzing a total of 150–250 myofibers.

For analyses of myonuclear number, sections from the belly of each muscle were rehydrated in PBS for 10 min and then incubated with anti-dystrophin antibody (1:400 in PBS + 5% goat serum) for 1 h at room temperature. Sections were washed with PBS + 0.1% Tween 20 (PBST) and then incubated with Texas Red-conjugated goat anti-mouse IgG (1:50 in PBS + 2% goat serum). After further washing in PBST, the sections were fixed in 2% formaldehyde for 10 min. The sections were again washed in PBST and incubated with a 0.25 μg/ml solution of the nuclear dye 4,6-diamidino-2-phenylindole (Sigma) for 10 min, washed again, and mounted in Vectashield mounting media. Nuclei that lie within the myofiber sarcolemma as defined by dystrophin staining were counted for 150–200 fibers per muscle, and the number of myonuclei was expressed per 100 myofibers.

CN activity assay and protein quantitation. Groups of 10 soleus or plantaris muscles were homogenized in 0.5 ml of lysis buffer from the Biomol Green Cellular Calcineurin Assay Kit Plus (Biomol Research Laboratories, Plymouth Meeting, PA). Muscle CN activity in muscle homogenates was determined as per the manufacturer’s protocol. In brief, muscle homogenates were centrifuged at 150,000 g and desalted using a P6 DG resin with a molecular mass exclusion limit of 6 kDa provided with the kit to remove excess phosphate and other nucleotides. Aliquots (5 μl) of desalted samples were added to assay buffer containing RII phosphopeptide as a substrate for CN in triplicate. Samples were then incubated at 30°C for 10 min, after which 100 μl of Biomol Green reagent were added to stop the reaction. The samples were then incubated at room temperature for 30 min to allow color development, and the optical density at 620 nm (OD620) was determined for each sample. CN-specific phosphatase activity was determined by subtracting the OD620 of samples with total phosphatase activity from the OD620 of samples in the presence of EGTA. The amount of free phosphate was determined by using a phosphate standard curve, which was performed in parallel with the samples. Total protein content of each soleus and plantaris group was determined in quadruplicate using the Bradford assay (9). CN activity was then normalized to CN-A protein levels in each sample as determined below.

CN protein levels in soleus and plantaris homogenates were determined by immunoblotting. Equal amounts of desalted muscle homogenate (50 μg/lane) were separated by
SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Burlington, MA). After nonspecific binding was blocked in 5% nonfat dry milk (NFDM) for 45 min, the membrane was incubated overnight at 4°C in 0.5% NFDM in TBS containing a 1:1,000 dilution of anti-CN-Aa/β (Chemicon International, Temecula, CA) or 1:2,000 dilution of anti-CN-B (Affinity BioReagents, Golden, CO). Blots were then washed extensively in TBS containing 0.1% Tween-20 (TBS-T) and then incubated with a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000) in 0.5% NFDM in TBS-T. Cyclophilin B (CyP-B) was detected in homogenates as above, except that a 1:2,000 dilution of anti-CyP-B (Affinity BioReagents) was used. Blots were washed in TBS-T, and antibody binding was detected using enhanced chemiluminescence. Blots were then scanned and quantified using Scion Image software. To verify equal loading and transfer of protein, membranes were stained with Coomassie Blue (Bio-Rad, Hercules, CA). Levels of CN-A, CN-B, and CyP-B were normalized by expressing the signal obtained from experimental lanes relative to a control soleus homogenate sample that was included with every immunoblot.

Statistics. To determine significance between two groups, comparisons were made using Student’s t-tests. Analyses of multiple groups were performed using one-way ANOVA with Bonferroni’s post test using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

RESULTS

Effects of CsA on normal muscle maintenance. To analyze the effects of CsA on maintenance of normal muscle size, Balb/c mice were treated with CsA at 12, 25, and 50 mg/kg for 2 wk. Subsequently, the cross-sectional area of myofibers was determined in the slow-twitch soleus and the fast-twitch plantaris as a measure of muscle size. Immunosuppression studies in Balb/c mice have shown that 10–50 mg/kg CsA administered once daily is therapeutic, whereas increasing the dose to 100 mg/kg daily is toxic (5, 22).

As shown in Fig. 1A, the cross-sectional area of soleus myofibers was not significantly decreased with respect to vehicle treatment at any of the three doses of CsA tested. The percentage of type I myofibers was also determined in these experiments and found not to significantly differ among treatment groups (data not shown). In contrast to the soleus, normal plantaris myofibers atrophied in a dose-dependent manner after 2 wk of CsA treatment, leading to an ~25% decrease in myofiber cross-sectional area at 50 mg/kg (Fig. 1B).

Taken together, these results show that myofiber size in the soleus and plantaris is differentially affected by CsA treatment, possibly reflecting differences in molecular mechanisms responsible for maintenance of muscle size in phenotypically different muscles.

Effects of CsA on growth of the soleus and plantaris muscles after atrophy. We next examined whether CsA affects growth of atrophied soleus and plantaris muscles. Atrophy was induced by 2 wk of hindlimb suspension, which we previously demonstrated leads to 51% and 23% atrophy of soleus and plantaris myofibers, respectively (25). Mice were treated with CsA for 2 wk after HS, starting on the same day the atrophic stimulus was removed. In vehicle-treated mice, both muscles grew to control values during this 2-wk period. CsA at 12 mg/kg did not significantly attenuate growth of soleus myofibers, whereas the 25 and 50 mg/kg doses inhibited growth by ~23% and ~50%, respectively (Fig. 2A). In contrast, CsA treatment resulted in a
further demonstrate differential effects of CsA on skeletal muscle growth depending on muscle type.

Myonuclear number of normal and recovering soleus and plantaris myofibers. Myonuclear accretion is necessary for growth of the atrophied soleus (25). Because CN is necessary for differentiation of MPCs in vitro (13, 18), we analyzed whether CsA affects the number of nuclei within myofibers.

First, normal mice treated for 2 wk with CsA were analyzed. Myonuclear number of soleus or plantaris myofibers was not significantly altered by CsA in these mice at all three doses tested (Fig. 3A and B). Subsequently, we analyzed myonuclear number in recovering muscles of mice treated with CsA for 2 wk. Here, soleus myonuclear number was significantly decreased by 50 mg/kg CsA (Fig. 3A). Because the myonuclear number of plantaris myofibers did not decrease concomitantly with the 23% decrease in myofiber cross-sectional area after 2 wk HS (25), we did not expect any difference with CsA treatment during recovery. At 12 and 25 mg/kg CsA, myonuclear number was indeed not altered. Surprisingly, however, 2-wk treatment with 50 mg/kg CsA resulted in a significant decrease in myonuclear number of plantaris myofibers. Possibly,
the 23% atrophy of the plantaris observed after 2 wk HS was not enough to elicit a decrease in myonuclear number; however, the 32% atrophy caused by 2-wk treatment with CsA during recovery was sufficient to elicit a decrease in myonuclear number to conserve a constant myonuclear domain. These data suggest that the effects elicited by CsA on myofiber size in the soleus at 50 mg/kg may be due, at least in part, to preventing the addition of myonuclei to growing myofibers. In contrast, in the plantaris where growth after atrophy is not dependent on myonuclear accretion (25), CsA elicits its effect independently of MPC. Thus CsA appears to affect muscle growth by multiple cellular mechanisms.

Short-term CsA treatment reveals CN-dependent and CN-independent mechanisms of growth. To examine further the mechanisms by which CsA inhibits growth of the soleus, experiments using short-term administration of CsA were performed. We previously demonstrated that the growth of the atrophied soleus in the first week occurs independently of myonuclear accretion (25). Thus CsA treatment during the first week of growth is most likely to affect myofiber-dependent processes.

Mice were treated with 50 mg/kg CsA for the first week after HS. We also gamma irradiated one of the hindlimbs to eliminate MPC and block myonuclear accretion (25) and used the contralateral hindlimb as the control. At 1 wk of recovery, 50 mg/kg CsA did not significantly decrease the cross-sectional area of soleus myofibers, either in gamma-irradiated or nonirradiated muscles (Fig. 4A). Soleus myofibers grew normally compared with vehicle-treated controls in the presence of CsA, such that 50% of myofiber cross-sectional area was regained after 1 wk of recovery. These results suggest that the first 50% of soleus growth occurs independently of CN-mediated pathways. Thus growth of the atrophied soleus muscle is controlled by a mixture of CN-independent and CN-dependent pathways that come into play at different times during the course of muscle growth.

Similar studies were performed on the plantaris muscle in the same animals. Because the plantaris muscle does not lose myonuclei during HS and growth after atrophy is not dependent at any stage on myonuclear accretion (25), analysis of the plantaris in the experiment is only useful in defining whether a shorter treatment with CsA also induces further loss of cross-sectional area over that obtained with HS alone (Fig. 2B). Plantaris myofibers recovered 85% of the cross-sectional area lost during HS after 1 wk of recovery (Fig. 4B). In the presence of CsA, however, plantaris myofibers did not grow and remained similar in size to those after 2 wk of HS. As expected from our previous study (25), gamma irradiation had no effect on recovery of the plantaris in vehicle- or CsA-treated mice. Thus the majority of plantaris growth occurs in the first week of recovery and appears to be controlled by a CN-dependent pathway. Longer treatment with CsA is required to obtain the observed atrophy over that induced by HS alone. Taken together, these results further suggest that disparate muscle types utilize CN-dependent pathways of growth to different extents.

Expression of regulatory proteins involved in CN signaling pathway. The disparity between the effects of CsA on both normal muscle maintenance and growth of the soleus vs. plantaris prompted biochemical analyses of the CN regulatory pathway. CN forms a heterodimer consisting of A and B subunits (32). CN-B regulates the activity of the catalytic subunit CN-A and is required for CsA-mediated inhibition of CN activity (20). In addition to modulation of CN-A protein levels, several possible mechanisms exist to regulate the activity of CN. One possible mechanism could be through regulation of CN-B protein levels. Another possible mechanism could be through regulation of expression of the various isoforms of cyclophilin (CyP), the intracellular receptor for CsA (21). Several studies have shown that the different isoforms of CyP (A, B, and C), when bound to CsA, bind and inhibit CN to different extents. Specifically, CyP-B complexed to CsA forms a highly active complex and inhibits CN to a greater extent than CyP-A and CyP-C (10, 35). We reasoned that possible
Differences in the expression of CN-B and the CyP isoforms between the soleus and plantaris might partly explain why the plantaris is more severely affected by CsA.

CN-A and CN-B protein levels were determined in soleus and plantaris homogenates by immunoblotting. Using an antibody to detect both α- and β-isoforms of CN-A, we showed that CN-A levels in the plantaris are 428% higher than in the soleus (Fig. 5A). These results are in agreement with a recent report showing that CN-A protein levels are higher in the plantaris (34). To detect CN-B protein levels, an antibody that recognizes both CN-B1 and CN-B2 isoforms was used. The plantaris contains 59% less CN-B than the soleus (Fig. 5B), indicating that the plantaris has less CN-B per molar equivalent of CN-A compared with the soleus. Because CN-B is required for phosphatase activity (19, 24, 30), these results suggest that there is a smaller proportion of total CN-A that is capable of being enzymatically active in the plantaris. Next, we analyzed CyP-B protein levels in control soleus and plantaris muscles, since CyP-B:CsA forms the most active complex in terms of inhibition of CN activity (35). CyP-B protein levels in the plantaris are 275% higher than in the soleus (Fig. 5C). These data indicate that CN activity in the plantaris may be more susceptible to the effects of CsA, due to the levels of CyP-B.

**CN activity in soleus and plantaris muscles.** Differential expression of regulatory proteins may lead to differences in CN activity levels. Such differences in CN activity would be hypothesized to play a role in determining a muscle’s response to CsA treatment. CN activity was determined in soleus and plantaris homogenates from mice treated with increasing doses of CsA for 1 wk. CN activity was then normalized to both protein content and relative protein levels of the catalytic CN-A subunit (α- and β-isoforms) from each homogenate. CsA at 12 mg/kg had no effect on CN activity in the soleus; however, 25 and 50 mg/kg CsA decreased CN activity by ~50% and ~70%, respectively (Fig. 6A). These data correlate well with the effects of CsA on soleus myofiber cross-sectional area. Specifically, 12 mg/kg CsA did not alter the cross-sectional area of myofibers, whereas increasing doses of CsA resulted in significantly decreased cross-sectional area. CN activity in the vehicle-treated plantaris is ~50% of the activity observed in the soleus (Fig. 6B). No CN activity was detected in plantaris muscles from 12, 25, or 50 mg/kg CsA treated mice. Low levels of CN activity may not be observed in the CsA-treated plantaris because of the sensitivity limits of the assay. Importantly, these results demonstrate that the higher CN-A protein levels in the plantaris do not result in increased CN activity and partly explains why the plantaris is affected more by CsA.

**DISCUSSION**

CsA, an inhibitor of CN-dependent signaling pathways, differentially affects muscle size in the phenotypically distinct soleus and plantaris muscles. The fast-twitch plantaris is more severely affected by CsA than the slow-twitch soleus, both in terms of maintenance of normal muscle size as well as growth after atrophy, suggesting that disparate muscle types utilize CN-dependent pathways of growth to different extents.

The role of CN in regulating skeletal muscle growth is controversial. A number of factors may contribute to the often conflicting data in this field. The experimental approach used to modulate CN may be one such factor. The majority of studies use CsA without demonstrating that the degree of inhibition of CN activity correlates with the observed experimental outcomes. However, CsA can elicit effects via CN-independent mechanisms (3, 12, 37). Depending on the specific process under investigation, such CN-independent mechanisms may contribute a greater or lesser effect to the experimental outcome. Transgenic mice that express activated CN do not demonstrate muscle hypertrophy (28). These results may be construed as demonstrating that CN does not play a role in this form of muscle growth. Equally plausible explanations are either that the proper upstream signaling pathways (16) or downstream targets are limiting.

As highlighted by the results presented here, the dose of CsA as well as the length of treatment are also critical parameters to consider when analyzing the effects of CsA on muscle size. A recent study refutes the role of CN in muscle growth, based on results showing that CsA treatment for 1 wk did not inhibit growth of...
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The soleus after HS-induced atrophy (8). Those data are difficult to interpret, as the dose of CsA used in that study only minimally inhibited CN activity during overload (8). Furthermore, our results demonstrate that growth of the soleus during the first week is CsA insensitive followed by a later CsA-sensitive phase. In contrast, muscle regeneration is characterized by an early CsA-sensitive phase (1) and a later CsA-insensitive phase (33). Together, these results illustrate the importance of selecting appropriate time points when studying signaling pathways during muscle growth. Differences in gender, species (rat vs. mouse), or even strain differences in mice may also serve to explain some of the disparate results in the literature.

Muscle type may also be a major confounding variable when analyzing the role of CN in skeletal muscle growth. The majority of studies were performed in predominantly fast-twitch muscles (15, 34). Dunn et al. (15) demonstrated that 2–4 wk of treatment with CsA completely prevented hypertrophy of the plantaris in response to overload. In the vastus intermedius, CN levels and muscle mass were positively correlated after spinal cord transection (34). We show here that CsA not only completely prevents growth of the atrophied plantaris muscle but also leads to further atrophy. In contrast, growth of the soleus myofibers after HS-induced atrophy is attenuated by only 25–50% at these same doses. Finally, the mechanisms regulating increases in muscle size may differ depending on the specific type (hypertrophy, growth after atrophy, maintenance, regeneration) or stimulus (IGF-I vs. overload-induced hypertrophy) for muscle growth. The relative contribution of MPC vs. myofiber intrinsic processes may vary depending on the type of growth (4, 31). CN is involved in regulating MPC differentiation (13, 18), and growth that is heavily dependent on MPCs would be more sensitive to the abrogation of CN activity.

Our studies extend previous work in the area of CN and muscle growth by also analyzing the effects of CsA on maintenance of normal muscle size in phenotypically distinct muscles. Two-week treatment with CsA differentially affects the cross-sectional area of myofibers in normal soleus and plantaris muscles. Soleus myofiber cross-sectional area is not affected after 2 wk of CsA treatment. These data are in agreement with previous reports on the lack of long-term effects of CsA on soleus weight (7, 15). A different study reported decreases in soleus weight after 3 wk of CsA treatment; however, no myofiber cross-sectional area determinations were performed (6). Although CsA treatment did not affect normal soleus myofiber cross-sectional area in our study, components of the metabolic and contractile phenotype may be affected with CsA treatment. The effects of CsA on metabolic and contractile molecules in normal soleus muscle have been reported elsewhere (6). In contrast to our findings in the soleus, plantaris myofibers atrophy significantly after 2 wk of treatment with CsA. These data are at variance with another study (15) showing no effects of CsA on the normal plantaris. The reasons for this discrepancy are unknown but may reflect differences in gender and mouse strain used. The differential effects of CsA on the soleus and plantaris shown here on two different forms of muscle growth suggest that differences exist in the mechanisms regulating growth of these two muscles.

To determine why the soleus and plantaris react differently to the same concentrations of CsA, we investigated molecular components involved in the CN signaling pathway in each muscle. Specifically, levels of the CN catalytic and regulatory subunits as well as a particular CyP were compared in the two muscles. The catalytic subunit of CN, CN-A, is expressed at higher levels in the plantaris, consistent with recent reports (34, 36). However, the regulatory subunit of CN, CN-B, is expressed at lower levels in the plantaris. Because CN-B is required for activity of CN-A (19, 24, 30), these data suggest that the plantaris contains a smaller proportion of CN capable of being enzymatically active compared with the soleus. Recent reports also indicate that the CsA sensitivity of a cell is dependent on both the total levels and relative expression of the CN catalytic and regulatory subunits.

Fig. 6. Effects of CsA on CN activity in the soleus and plantaris. Mice were treated with CsA at the doses indicated for 1 wk. CN activity was normalized to protein content and relative levels of CN-A in each sample as determined by immunoblotting (data not shown). A: CsA attenuated CN activity in the soleus. B: CN activity in the plantaris was inhibited by all 3 doses of CsA tested. Data are means ± SE; n = 5, *P < 0.05.
levels of CyP-A, CyP-B, and CyP-C. All three CyPs have been shown to differ in the efficiency at which they inhibit CN when bound to CsA. In particular, CyP-B forms the most active complex capable of inhibiting CN when bound to CsA (35). Consistent with the differential effects of CsA reported here on both muscles, CyP-B levels are higher in the plantaris. Consequently, certain muscles may be protected from the effects of CsA by low levels of CyP-B, whereas other muscles may be more susceptible due to higher levels of CyP-B.

CN activity was determined in soleus and plantaris muscles from vehicle- or CsA-treated mice to determine whether the differences observed in the levels of regulatory proteins affect CN activity in either muscle. In agreement with the differences reported in CN-A and CN-B levels between the two muscles, CN activity in the vehicle-treated plantaris is ~50% of the activity measured in the soleus muscle under the same conditions. At higher doses of CsA, CN activity is decreased in the soleus, and no CN activity is detected in the plantaris with CsA treatment, possibly due to increased levels of CyP-B. These data demonstrate that CN activity in the plantaris is affected more by CsA and correlates well with the differential effects on myofiber cross-sectional area during normal maintenance. At higher doses of CsA, CN activity is decreased in the soleus, and no CN activity is detected in the plantaris with CsA treatment, possibly due to increased levels of CyP-B. These data strongly suggest that CN-dependent pathways within muscle itself are mainly responsible for the effects of CsA on muscle growth observed in the current study. Furthermore, these data show that increased levels of CN-A do not necessarily correlate with increased CN phosphatase activity. Taken together, these data suggest that CN-mediated signaling pathways in skeletal muscle may be regulated not only by inductive signals that increase intracellular Ca\textsuperscript{2+}, but also via a complex mechanism involving regulation of expression of CN subunits. Immunosuppression studies have shown that CsA does not completely abrogate CN activity, even at saturating levels of CsA (23), further suggesting that CN activity may ultimately be affected by molecular mechanisms that dictate the expression of regulatory proteins that interact and modulate the activity of CN. Furthermore, sensitivity of different muscles to CN-inhibiting drugs such as CsA may be dependent not only on the dose of CsA used, but also on the expression of its intracellular receptors.

Cellular mechanisms regulating the growth of the atrophied soleus and plantaris also differ (17, 25) and may contribute to the differential effects of CsA. Specifically, myonuclear accretion contributes to growth in the soleus, but not in the plantaris. In the current study, CsA significantly attenuates myonuclear accretion in soleus myofibers, suggesting that CsA affects growth of the atrophied soleus in part by inhibiting MPC differentiation and fusion. Such an effect would be consistent with the fact that both pharmacological and genetic inhibitors of CN block differentiation in vitro (13, 18). These data lend further support to our conclusions that CN-dependent effects of CsA are responsible for regulating muscle growth. Some myonuclei, however, are incorporated into growing soleus myofibers. Incomplete inhibition of CN activity may partly explain why myonuclear accretion is not completely prevented in growing soleus myofibers. In contrast, 2-wk treatment with 50 mg/kg CsA during growth of the plantaris significantly decreases myonuclear number. This is most likely due to the further atrophy that occurs after CsA treatment, resulting in myonuclear loss to conserve a constant myonuclear domain in plantaris myofibers (2).

In summary, CsA treatment affects muscle growth and maintenance differently, depending on muscle phenotype. CN activity appears to be required for muscle growth at specific stages during the growth process. Based on the results of the current study, together with those in Mitchell and Pavlath (25) examining the role of MPC in myofiber growth, we propose the following model of growth after HS-induced atrophy in the phenotype and functionally disparate soleus and plantaris (Fig. 7). In contrast to the plantaris, atrophy of soleus myofibers is accompanied by a loss of myonuclei. Soleus myofiber growth thereafter appears to be mediated by myofiber-intrinsic processes, independent of the CN signaling pathway. Later growth, which requires myonuclear accretion, is CN dependent. Growth of plantaris myofibers occurs without myonuclear accretion, suggesting that exclusively myofiber-dependent processes contribute to growth of the plantaris after atrophy. Both initial and later stages of its growth are inhibited by CsA, suggesting that this growth is dependent on CN activity.

This work demonstrates the complexity of mammalian skeletal muscle growth. Not only are different mechanisms of growth used by specific muscle types, but also at different stages within the same muscle. These findings should provide insight into the develop-
ment of rehabilitative strategies for enhancing growth in disease, disuse, and aging.

We thank Dr. Gordon Warren and Dwayne Blaylock for assistance with gamma irradiation experiments. This work was supported by National Institute of Health Grants AR-47314 and DE-13040 to G. K. Pavlath.

REFERENCES


