Calcineurin Co-regulates Contractile and Metabolic Components of Slow Muscle Phenotype*

Received for publication, January 20, 2000, and in revised form, March 20, 2000
Published, JBC Papers in Press, April 20, 2000, DOI 10.1074/jbc.M000430200

Xavier Bigard‡, Hervé Sanchez§, Joffrey Zoll‡, Phillipe Matteo¶, Vincent Rousseau‡, Vladimir Veksler†, and Renée Ventura-Clapier††

From the ‡Unité de Bioénergétique et Environnement, Centre de Recherches du Service de Santé des Armées, Avenue du Maquis du Grésivaudan, 38702, La Tronche Cedex, France, the §U-446 INSERM, Cardiologie Cellulaire et Moléculaire, Université Paris-Sud, 92296 Châtenay-Malabry, France, and ¶Service de Physiologie Clinique et des Explorations Fonctionnelles, Hôpitaux Universitaires de Strasbourg, 67091 Strasbourg Cedex, France

Activation of the transcription factor nuclear factor of activated T cells by the calcium-sensitive serine/threonine phosphatase calcineurin has been proposed as one of the molecular mechanisms by which motor nerve activity establishes the slow muscle phenotype. To investigate whether the calcineurin pathway can regulate the large spectrum of slow muscle characteristics in vivo, we treated rats for three weeks with cyclosporin A (an inhibitor of calcineurin). In soleus (slow muscle), but not in plantaris (fast muscle), the proportion of slow myosin heavy chain (MHC-1) and slow sarcoplasmic reticulum ATPase (SERCA2a) was decreased, whereas that of fast MHC (MHC-2A) and fast SERCA1 increased, indicating a slow to fast contractile phenotype transition. Cytosolic isoforms of creatine kinase and lactate dehydrogenase (most abundant in fast fibers), as well as mitochondrial creatine kinase and citrate synthase activities (elevated in fast/oxidative fibers) were dose dependently increased by cyclosporin A treatment in soleus muscle, with no change in plantaris. Calcineurin catalytic subunit was more abundant in soleus muscle fibers compared with plantaris. Taken together these results suggest that the calcineurin pathway co-regulates a set of multigenic protein families involved in the transition between slow oxidative (type I) to fast oxidative (type IIa) phenotype in soleus muscle.

The functional and structural diversity of mammalian skeletal muscles is met by skeletal muscle fibers differing in their morphological, biochemical, and contractile properties and their assembly in various proportions. Four major fiber types have been characterized according to their contractile and metabolic properties: slow oxidative (type I), fast oxidative/glycolytic (type IIX and IIb) fibers, with no change in plantaris. Calcineurin catalytic subunit was more abundant in soleus muscle fibers compared with plantaris. Taken together these results suggest that the calcineurin pathway co-regulates a set of multigenic protein families involved in the transition between slow oxidative (type I) to fast oxidative (type IIa) phenotype in soleus muscle.

* This work was supported by Association Francaise contre les Myopathies and by the PROGRES program from INSERM. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Centre National de la Recherche Scientifique. To whom correspondence should be addressed: U-446 INSERM, Cardiologie Cellulaire et Moléculaire, Université Paris-Sud, 5 rue J-B Clément, 92296 Châtenay-Malabry, France. Tel.: 33 1 46 83 57 62; Fax: 33 1 46 83 54 75; E-mail: Renee.Ventura@cep.u-psud.fr.

1 The abbreviations used are: NFAT, nuclear factor of activated T cells; MHC, myosin heavy chain; CsA, cyclosporin A; SERCA, sarcoplasmic reticulum Ca²⁺ ATPase; CR, creatine kinase; LDH, lactate dehydrogenase; mi-CR, mitochondrial CK; SOL, soleus; PL A, plantaris; CS, citrate synthase; Vh, vehicle; MES, 4-morpholinoethanesulfonic acid; CnA, calcineurin; CD, capillary density; CF, capillary to fiber ratio; C, control.
metabolism than slow oxidative fibers, whereas IIX and IIB fibers have lower oxidative potential (13). Thus, either an increase or a decrease in oxidative enzymes is expected with a slow to fast transition, depending on the extent of the transition. We recently reported that oxidative capacity of in situ mitochondria of soleus and plantaris muscle fibers was not altered by CsA itself but by its vehicle (14), whereas effects of CsA per se on the activity of specific mitochondrial markers have not been investigated so far. Furthermore, other enzymes such as creatine kinase (CK) and lactate dehydrogenase (LDH), two important families of metabolic enzymes, exist as multiple isoforms contributing to the molecular diversity of muscle fibers. M-CK and M-LDH activities are elevated in fast fibers, whereas the sarcomeric mitochondrial CK (mi-CK) and H-LDH are high in oxidative fibers. As the mi-CK gene possesses the NFAT recognition sequence in its 5’-end, this renders it a possible candidate for regulation by calcineurin, although no data are yet available (4). A correlation between the LDH and CK isozyme profiles and the anaerobic/aerobic glycolysis capacity of muscle has been shown during muscle unloading (15) suggesting a possible regulation by calcineurin.

Finally, the capillary bed provides the final pathway for delivery of oxygen and substrates to muscle fibers. A relationship between capillary length and mitochondrial volume density was found in several species (16). This hypothesis is supported by the parallel increase in capillary network and oxidative capacity with electrical stimulation (17) and decreased capillarity with deconditioning (18). A decrease in the capillary bed has been observed in a fast twitch muscle in rats treated with low doses of CsA (7).

The purpose of the present study was thus to investigate whether the calcineurin pathway is active in vivo to maintain slow fiber phenotype and whether it is involved in the fiber type transition from fast IIB ⇒ IIX ⇒ IIa ⇒ slow I or is restricted to a given stage of phenotype. Moreover, as the generation of functional and highly specialized myofibers requires the coordinated regulation of different proteins during fiber type transitions, we tested whether calcineurin inhibition by CsA treatment mimics the effects of decreased motor nerve activity on morphological, metabolic, and contractile characteristics of slow muscle. The purpose of the present study was thus to investigate the effects of in vivo inhibition of the calcineurin pathway by CsA treatment in both a slow twitch (soleus, SOL) and a fast twitch (plantaris, PLA) skeletal muscle on 1) the oxidative capacity with electrical stimulation (17) and decreased capillarity with deconditioning (18). A decrease in the capillary bed has been observed in a fast twitch muscle in rats treated with low doses of CsA (7).

The purpose of the present study was thus to investigate whether the calcineurin pathway is active in vivo to maintain slow fiber phenotype and whether it is involved in the fiber type transition from fast IIB ⇒ IIX ⇒ IIa ⇒ slow I or is restricted to a given stage of phenotype. Moreover, as the generation of functional and highly specialized myofibers requires the coordinated regulation of different proteins during fiber type transitions, we tested whether calcineurin inhibition by CsA treatment mimics the effects of decreased motor nerve activity on morphological, metabolic, and contractile characteristics of slow muscle. The purpose of the present study was thus to investigate the effects of in vivo inhibition of the calcineurin pathway by CsA treatment in both a slow twitch (soleus, SOL) and a fast twitch (plantaris, PLA) skeletal muscle on 1) the oxidative capacity with electrical stimulation (17) and decreased capillarity with deconditioning (18). A decrease in the capillary bed has been observed in a fast twitch muscle in rats treated with low doses of CsA (7).

Tissue Processing—Following three weeks of treatment, animals were anesthetized with sodium pentobarbital (90 mg/kg body weight) administered intraperitoneally. SOL and PLA muscles were excised, cleaned of adipose and connective tissue, and weighed. Muscles of the right hindlimb were mounted in an embedding medium (TEK O.C.T. compound) and frozen in isopentane cooled to the freezing point (−160 °C) by liquid nitrogen, whereas muscles of the left hindlimb were weighed and immediately frozen in liquid nitrogen for biochemical determinations. Both dorsal and epidymidal fat pads were excised, cleaned, and weighed. The tibia length was measured in the right leg after dissection. All samples were stored at −80 °C until histochemical and biochemical analyses were performed.

In situ histochemistry—Transverse sections (10 μm thick) were cut from the mid-belly portion of SOL and PLA in a cryostat maintained at −20 °C. Some sections were labeled with mouse monoclonal antibodies against myosin reacting either with 1) slow type 1 (Novocastra, reference NCL-MHCS, Newcastle upon Tyne, UK), 2) all adult fast and developmentally regulated epitopes but not with slow myosin (MY-32, Sigma), 3) fast type 2A (SC-71), 4) slow and fast type 2A (SC-13390), and 5) slow type 2B (SC-71), but not with type 1 myosin (Novocastra, reference NCL-MHCS, Newcastle upon Tyne, UK). The catalytic subunit of calcineurin, designated PP2B-A, was localized in SOL and PLA muscles after incubation with a rabbit polyclonal antibody (SC9070, Biophore Biotechnology, Tous, France). This antibody was also used to label sections for 12 h at +4 °C. The avidin-biotin immunohistochemical procedure was used for the localization of the antigen-antibody binding (Vector Laboratories, Burlingame, CA). Negative control slides with omission of the primary antibodies were randomly included in the immunostaining procedure. A sample of ~400 fibers was randomly selected from fields equally distributed over the biopsies for single fiber MHC composition. Fibers were classified according to their staining profile with the aid of a microscope linked to a computer-based image analysis system (Visiolab 200, Nikon-France). Moreover, some sections were stained with hematoxylin eosin to evidence the nuclei (19).

Analysis of MHCs—Muscles were subjected to the analysis of MHC isoforms as described previously (20). Myosin was extracted and separated in acrylamide gel solution containing 30% glycerol, 8% acrylamide, 0.2 M Tris, 0.1 M glycine, and 0.4 M sodium dodecyl sulfate (SDS). Electrophoresis was performed using a Mini Protein II system (Bio-Rad). Gels were run at constant voltage (70 V) for ~28 h and then stained with Coomassie Blue. The MHC protein isoform bands were scanned and quantified by using a densitometer system equipped with an integrator (GS-700, Bio-Rad).

Biomechanical Determinations—Frozen tissue samples were weighed and placed into an ice-cold homogenization buffer (30 mg wet weight/ml containing; 5 mM Hepes (pH 8.7), 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl2, and 0.1% Triton. Samples were homogenized using a micro-glass hand homogenizer and were incubated for 60 min at 0 °C to ensure complete enzyme extraction. The total adenylyl kinase and CK activities were assayed as described previously (15). CK isoenzymes were separated using agarose (1%) gel electrophoresis performed at 200 V for 90 min. To avoid saturation of the various CK isoforms, three dilutions were used for each sample. Individual isoenzymes were resolved by incubating the gel with a paper soaked with staining solution containing: 22 mM MES (pH 7.4), 50 mM magnesium acetate, 70 mM glucose, 120 mM n-acetyl cysteine, 9 mM ADP, 120 mM phosphocreatine, 9 mM NADP, 0.1 mM P1, P5-diadenosine-5’-pentaphosphate (to inhibit adenylate kinase), 9 IU/ml hexokinase, and 6 IU/ml glucose-6-phosphate dehydrogenase. Isoenzyme bands were visualized and quantified using an image analysis system (Bio-Rad). The LDH isoenzyme profile was determined using agarose gel electrophoresis (Sigma LDH reagent kit, Sigma) at 200 V for 90 min followed by image analysis. CS activity was determined according to Ref. 21.

Capillary Staining—Capillaries were visualized using acidic adenosine triphosphatase (ATPase) reaction (22) and identified using a computer-based image analysis system (Visiolab 200, Nikon-France). Four to six areas were selected on each sample and they represented total area A. These areas were randomly determined on the expanse of the SOL and PLA muscles. The capillary bed was appraised according to the following parameters: 1) the capillary density (CD), which was calculated as the number of capillaries in the total area (A) divided by the area of A; and 2) the capillary to fiber ratio (CF), which was determined as CD normalized by fiber density where fiber density

MATERIALS AND METHODS

Animals—Male Wistar rats initially weighing 180 g were purchased from IFFA Credo (L’Arbresle, France). Animals were housed 4/cage in a thermoneutral environment (22 ± 2 °C) on a 12:12 h photoperiod and were provided with food and water ad libitum. This investigation was carried out in accordance with the Helsinki Accords for Humane Treatment of Animals during Experimentation.

Experimental Design—After 3 days acclimatization to the animal room, rats were randomly assigned to one of four experimental groups. The animals received orally either a daily dose of 10 (n = 7) or 25 (CsA group, n = 8) mg/kg CsA as Sandimmum® diluted in 0.5 ml of olive oil, a daily dose of 0.5 ml of the vehicle (Vh, n = 8), or a daily dose of 0.5 ml of water to have a control of the gavage procedure (C, n = 7). The complete vehicle of Sandimmum® was reconstituted from two-thirds Cremophor® EL (BASF, Germany) and one-third alcohol diluted in olive oil. Vh animals received a volume of vehicle equivalent to that of a daily dose of 25 mg/kg CsA. Doses of Sandimmum® and vehicle were adjusted according to body weight gain.
The mean number of fibers/mm² on the border line of the area were counted (22).

**RESULTS**

**Blood Cyclosporin Levels**—Blood CsA levels have been measured in 7 rats treated with 10 mg/kg/day of CsA and 5 animals treated with 25 mg/kg/day. As expected, the mean cyclosporin level in blood was lower in the former than in the latter (742 ± 199 ng/ml and 1896 ± 311 ng/ml, respectively, p < 0.02). A large scattering of CsA levels was found reflecting the great variability in the bio-availability of the drug. This inter-individual variability was used to estimate the correlation between the blood level of CsA and parameters of interest, whereas mean values reported in the tables refer to the group treated with 25 mg/kg/day CsA (CsA group).

**Body and Muscle Mass**—Initial body weights did not significantly differ among groups. Three weeks of CsA treatment resulted in a lower body weight compared with the C and Vh groups (−33% and −23%, respectively, p < 0.01), whereas vehicle by itself was responsible for a 12% decrease in body weight (p < 0.01) (Table I). Following the three weeks treatment, the weight gain of the animals was inversely correlated with the blood level of cyclosporin (p < 0.0001, Fig. 1A). Both vehicle and CsA treatments slightly reduced the skeleton growth rate as tibia length was decreased by 3% and 5%, respectively (p < 0.01) (Table I). As a consequence, cyclosporin per se decreased the skeleton growth rate by 2% (p < 0.05).

The absolute weights of SOL and PLA muscles were affected by both the vehicle (−15%, p < 0.05 and −18%, p < 0.01, respectively) and the cyclosporin per se (−33%, p < 0.01 and −20%, p < 0.01, respectively). When normalized to tibia length, the SOL and PLA muscle weights were only affected by CsA (−31% and −18%, respectively, in comparison with Vh group, p < 0.01) (Table I). Both the absolute and the normalized fat pad weights of the CsA group were reduced relative to the C and Vh groups (−34%, p < 0.01 and −30%, p < 0.05, respectively). Muscles and fat weights were inversely correlated with blood CsA (p < 0.01) (Fig. 1B and C).

**MHC and Fiber Type Distribution in SOL and PLA Muscles**—Myosin heavy chains are the hallmark of fiber type. CsA treatment specifically affected the MHC profile of SOL as measured using gel electrophoresis. The MHC-1 relative content was lower in SOL muscles of CsA treated than Vh and C

|                  | C group | Vh group | CsA group | ANOVA   |
|------------------|---------|---------|-----------|---------|
| Final body weight, g | 324 ± 5 | 285 ± 5*** | 218 ± 8*** **** | p < 0.001 |
| Tibia length, cm  | 3.86 ± 0.01 | 3.73 ± 0.03** | 3.66 ± 0.02*** **** | p < 0.001 |
| Absolute muscle weight, mg | | | | |
| SOL               | 126 ± 7 | 107 ± 5* | 72 ± 4** **** | p < 0.001 |
| PLA               | 271 ± 9 | 221 ± 14** | 178 ± 8*** **** | p < 0.001 |
| Relative muscle weight, mg/cm |
| Soleus            | 32.7 ± 1.7 | 28.7 ± 1.2 | 19.7 ± 1.1** **** | p < 0.001 |
| Plantaris         | 70.2 ± 2.2 | 59.1 ± 3.4 | 48.7 ± 1.9** **** | p < 0.001 |
| Fat pad weight, g | 4.64 ± 0.36 | 4.04 ± 0.36 | 3.06 ± 0.29** **** | p < 0.01 |
| Relative fat pad weight, g/cm | 1.20 ± 0.09 | 1.08 ± 0.09 | 0.84 ± 0.08** | p < 0.05 |
Calcineurin and Slow Muscle Phenotype

TABLE II

| Table II | MHC distribution based on gel electrophoresis in soleus and plantaris muscles from C, Vh- and CsA-treated animals |
|----------|------------------------------------------------------------------------------------------------------------------|
|          | Values are mean ± S.E. in percentage of total myosin. Significant difference from C groups, *, p < 0.05; ***, p < 0.001. Significant difference from Vh group, ***, p < 0.001. ND, not detectable; NS, not significant. |
|          | C group | Vh group | CsA group | ANOVA |
| Soleus   |         |         |          |       |
| MHC-1    | 98.1 ± 0.9 | 96.6 ± 2 | 88.6 ± 2.5*** | p < 0.01 |
| MHC-2A   | 1.9 ± 0.9  | 3.4 ± 2  | 10.5 ± 2.6* ** | p < 0.05 |
| MHC-2X   | ND       | ND       | 0.9 ± 0.5*** | p < 0.05 |
| Plantaris|          |         |          |       |
| MHC-1    | 3.5 ± 1.1 | 2.7 ± 0.7 | 1.8 ± 1.6 | NS |
| MHC-2A   | 8.8 ± 1.3 | 5.7 ± 1.1 | 5.4 ± 1.6 | NS |
| MHC-2X   | 25.2 ± 2.7 | 27.6 ± 3.4 | 23.1 ± 4.1 | NS |
| MHC-2B   | 62.4 ± 3.7 | 63.9 ± 3.4 | 69.8 ± 4.3 | NS |

Table III

| Table III | Fiber type composition of soleus and plantaris muscles from C, Vh- and CsA-treated animals and percentages of fibers expressing or coexpressing SERCA isoforms |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|           | Values are mean ± S.E. in percentage. Type I fibers are those expressing only MHC-1, type IIA fibers are those expressing only MHC-2A, type IIB fibers are those expressing only MHC-2B. In SOL, type I/IIa fibers are those coexpressing both MHC-1 and MHC-2A. Significant difference from C groups, *, p < 0.05; ***, p < 0.001. Significant difference from Vh group, ***, p < 0.001; ***, p < 0.005, NS, not significant. |

|           | C group | Vh group | CsA group | ANOVA |
|-----------|---------|---------|----------|-------|
| Soleus    |         |         |          |       |
| Type I    | 90.5 ± 2.5 | 87.8 ± 3.8 | 62.8 ± 4.3*** | p < 0.01 |
| Type IIA  | 2.9 ± 1.1  | 2.1 ± 1.5  | 10.7 ± 3.5*** | p < 0.001 |
| Type IIB  | 6.6 ± 0.6  | 10.2 ± 3.1  | 36.4 ± 4.1*** | p < 0.001 |
| SERCA2a   | 89.4 ± 2.9  | 92.1 ± 1.7  | 73.6 ± 3.6*** | p < 0.005 |
| SERCA1    | 11.2 ± 1.8  | 8.2 ± 1.3  | 32.6 ± 2.8*** | p < 0.001 |
| Plantaris |         |         |          |       |
| Type I    | 10.5 ± 1.4  | 10.4 ± 1.6  | 8.5 ± 0.8  | NS |
| Type IIA  | 36.6 ± 1.6  | 34.7 ± 2.1  | 31.3 ± 2.4 | NS |
| Type IIB  | 34.4 ± 1.9  | 34.7 ± 1.3  | 31.4 ± 1.9*** | p < 0.001 |
| Type IIb  | 31.5 ± 2.2  | 30.4 ± 2.8  | 46 ± 2.8*** | p < 0.001 |

FIG. 2. Immunohistochemical detection of MHC-1 (A and E), MHC-2A (B and F), SERCA2a (C and G), and SERCA1 (D and H) within soleus muscles from rats treated with either vehicle (Vh group, A–D) or 25 mg/kg/d CsA (CsA group, E–H). Note the marked decrease in fibers expressing MHC-1 in SOL muscles of CsA group (E versus A), balanced by the increase in the percentage of fibers expressing MHC-2A (F versus B). Note also the strong coexpression of MHC-1 with SERCA2a isoform in Vh and CsA groups (A versus C and E versus G, respectively), and the coexpression of MHC-2A with SERCA1 isoform (B versus D and F versus H, respectively). Arrowheads denote fibers coexpressing MHC-1 with MHC-2A and SERCA2a with SERCA1. Calibration bar in D, 100 μm.

I fibers (p < 0.01) and a higher percentage of type IIA fibers (p < 0.01) in SOL, compared with both Vh and C rats (Fig. 2 and Table III). Moreover, the percentage of hybrid fibers containing both type I and type IIA MHC was higher in CsA group compared with those in C and Vh groups (p < 0.01). No pure type IIB fibers were detected in the CsA group, and the antibody against MHC-2B, the fastest MHC isoform, gave undetectable staining in all groups. As blood CsA increased, the total number of fibers expressing MHC-2A increased (Fig. 3A).

Only subtle changes in the fiber type distribution were shown in PLA muscles. There was a significant decrease in the percentage of type IIB fibers in CsA rats, compared with those in C and Vh groups (p < 0.005). Moreover, CsA rats had a higher percentage of type IIB fibers than both C and Vh groups (p < 0.001 and p < 0.005, respectively). No effect of vehicle per se was observed on MHC expression or fiber type distribution.

Immunocytdetection of SERCA Proteins in SOL Muscle—Because the cyclosporin-induced slow to fast transition in the MHC isoforms was mainly observed in SOL, the expression of specific SERCA isoforms in single fibers was only examined in this muscle. Vehicle did not affect SERCA expression (Table III). In normal SOL muscles, only 8% of fibers were stained with the SERCA1-specific antibody (Fig. 2). Fibers negative for SERCA1 stained for SERCA2a, and only few fibers expressed both isoforms. After 3 weeks of CsA administration, the percentage of SERCA1-positive fibers increased three times compared with both C and Vh groups (p < 0.05) (Fig. 2 and Table III). In SOL muscles of rats from the CsA group, the proportion of fibers expressing SERCA2a decreased to 71% compared with C and Vh groups (p < 0.05). A strong correlation was observed between the proportion of fibers expressing SERCA1 and blood CsA (Fig. 3B). Interestingly, a strong correlation was found between the percentage of fibers expressing MHC-2A and those expressing SERCA1 (p < 0.0001), as well as between the percentage of fibers expressing MHC-1 and those containing SERCA2a (p < 0.0001) isoforms in all SOL muscles (Fig. 4, A and B). Moreover, fibers coexpressing SERCA1 and SERCA2a were also those coexpressing MHC-1 and MHC-2A (Fig. 2).

Biochemical Properties—CsA treatment resulted in a 28% increase in the total LDH activity in SOL muscles (p < 0.05, Table IV). Moreover, the LDH isoenzyme composition of SOL muscles was also affected by the treatment because both the percentage (+26%, p < 0.01) and the activity (+63%, p < 0.01) of the M subunit measured in CsA group increased, compared with both C and Vh rats, with no change in the H subunit. Again, no significant change was observed in PLA muscles.

The creatine kinase system, involved in cellular energy transfer, is also a marker of the metabolic phenotype of muscle fibers. CsA increased total CK activity in SOL muscles, relative to C and Vh groups (p < 0.01) (Table IV). The specific activity...
of the cytosolic MM-CK isoenzyme increased in the SOL muscles of the CsA group in comparison with both C and Vh groups (131% and 136%, respectively, in a CsA-dependent manner (Fig. 3C). mi-CK tended to be higher in CsA group and was highly positively correlated with blood CsA (r = 0.73, p < 0.007).

Citrate synthase, an enzyme of the tricarboxylic acid cycle, is a marker of mitochondrial content in muscle. A significant increase in CS activity was observed in SOL muscles of the CsA group, in comparison with both C and Vh groups (65% and 45%, respectively, p < 0.01) (Table IV). These changes correlated well with the blood level of cyclosporin (p < 0.02). Vehicle had no effect per se on the expression of proteins involved in energy metabolism.

Skeletal Muscle Capillarity—SOL muscle exhibits a higher C/F than plantaris (Table V). CsA treatment induced a decrease in C/F in SOL muscles compared with the Vh group (−10%, p < 0.05). The increase in CD (62%) is likely related to the decrease in muscle weight and the associated decrease in the mean fiber cross-sectional area. An increase in CD was observed in PLA from the CsA group in comparison with C animals (13%, p < 0.05), but no significant change in C/F was detected.

Immunodetection of Calcineurin—Immunohistochemical localization of the catalytic subunit of calcineurin (CnA) has been performed in PLA and SOL muscles of vehicle-treated animals. In SOL muscle, CnA appears highly expressed in the periphery of the slow fibers and co-localized with or around the nuclei (Fig. 5, F and H). In contrast, more scarce labeling was observed in fast fibers of PLA muscle (Fig. 5, E and G).

**DISCUSSION**

The main results of this study can be summarized as follows. 1) CsA, an inhibitor of calcineurin, dose dependently decreased rat growth in a nontissue-specific manner suggesting the involvement of a calcineurin pathway in animal growth. 2) CsA induced a shift in glycolytic enzymes, SERCA, and contractile proteins of slow twitch skeletal muscle toward the fast twitch phenotype, in a muscle type-specific manner. 3) CsA dose dependently increased citrate synthase, and mi-CK activities, two markers of muscle oxidative capacity. 4) CsA affected the capillary bed of slow muscle as estimated by the capillary to fiber ratio. 5) Immunodetection of calcineurin showed that the catalytic subunit is present at higher levels in slow than fast twitch muscle. These results suggest that the calcineurin pathway regulates the transcription of numerous members of muscle-specific gene families and is involved in the transition from fast oxidative toward slow oxidative muscle phenotype in soleus muscle.

**Animal Growth**—Both vehicle and CsA affected the weight and growth of the animals. Previous studies showed that body growth of rats decreased after 3 weeks of treatment with 15 mg/kg/d CsA (23), whereas treatment with 7/mg/kg/d had no detectable effect (7), in accordance with the dose-dependent effect of CsA on animal growth (Fig. 1). The strong correlation between cyclosporin blood levels and relative mass of slow as well as fast muscles shows that skeletal muscle growth is under the control of the calcineurin pathway, as suggested both for the hypertrophic growth of heart and for overloaded skeletal muscle (8, 24). Effects of cyclosporin on growth are not limited...
Energy metabolism in soleus and plantaris muscles from C, Vh- and CsA-treated animals

Values are mean ± S.E. AK, adenylyl kinase; mi-CKCS, ratio of the activity of the mitochondrial CK isofrom to citrate synthase activity; M subunit %, percentage of the M subunit. Enzyme activities are expressed as units of activity/gram of tissue wet weight (IU · g⁻¹). Significant difference from both C and Vh groups, *, p < 0.05; **, p < 0.01. NS, not significant.

| SOL       | C     | Vh    | CsA   | ANOVA   |
|-----------|-------|-------|-------|---------|
| CS        | 33.1 ± 4.5 | 37.6 ± 1.6 | 54.7 ± 1.6 ** | p < 0.01 |
| AK        | 298 ± 55   | 250 ± 44   | 307 ± 56   | NS       |
| Total CK  | 1397 ± 125 | 1346 ± 38  | 1829 ± 89 ** | p < 0.005 |
| MM-CK     | 1349 ± 120 | 1300 ± 34  | 1769 ± 83 ** | p < 0.001 |
| mi-CK     | 47.6 ± 7.1  | 46.7 ± 6.2  | 59.6 ± 6.9  | NS       |
| Total LDH | 168 ± 20   | 167 ± 4    | 215 ± 13 *  | p < 0.05  |
| M subunit | 36 ± 1      | 34 ± 2      | 43 ± 2 * *  | p < 0.005 |
| Activity H subunit | 107 ± 12 | 110 ± 2    | 122 ± 6    | NS       |
| Activity M subunit | 61 ± 8    | 57 ± 4     | 93 ± 8 * *  | p < 0.005 |

| PLA       | C     | Vh    | CsA   | ANOVA   |
|-----------|-------|-------|-------|---------|
| CS        | 39.1 ± 2.9 | 40.9 ± 2.7 | 35.2 ± 2.1 | NS       |
| AK        | 905 ± 122 | 792 ± 144 | 628 ± 122 | NS       |
| Total CK  | 3416 ± 62 | 3381 ± 74 | 3228 ± 135 | NS       |
| MM-CK     | 3387 ± 63 | 3466 ± 75 | 3207 ± 135 | NS       |
| mi-CK     | 28.9 ± 3.1 | 24.3 ± 3.1 | 21.6 ± 1.6 | NS       |
| Total LDH | 724 ± 23   | 752 ± 12   | 744 ± 18   | NS       |
| M subunit | 78 ± 2      | 77 ± 1      | 77 ± 1     | NS       |
| Activity H subunit | 154 ± 12 | 177 ± 9    | 170 ± 10   | NS       |
| Activity M subunit | 509 ± 28 | 577 ± 15   | 571 ± 15   | NS       |

| TABLE V | Muscle fiber capillarity |
|---------|-------------------------|
|         | C group | Vh group | CsA group | ANOVA   |
| **Soleus** |         |         |           |         |
| Capillary density, (mm²)⁻¹ | 661 ± 26 | 982 ± 49 ** | 1020 ± 66 ** | p < 0.001 |
| Capillary to fibre ratio | 2.37 ± 0.09 | 2.55 ± 0.09 | 2.39 ± 0.08 *** | p < 0.01 |
| **Plantaris** |         |         |           |         |
| Capillary density, (mm²)⁻¹ | 762 ± 19 | 718 ± 30 | 864 ± 36 *** | p < 0.01 |
| Capillary to fibre ratio | 2.04 ± 0.05 | 1.95 ± 0.16 | 1.85 ± 0.08 | NS       |

Values are mean ± S.E. Significant difference from C groups, *, p < 0.05; **, p < 0.01. Significant difference from Vh group, ***, p < 0.05. NS, not significant.

Calcineurin and Slow Muscle Phenotype

The calcineurin/NFAT pathway is involved in the growth of animals in a non-tissue-specific manner.

Contractile Phenotype—There is a large body of evidence that low frequency electrical stimulation or nerve activity induce a fast to slow shift in the expression of contractile proteins (for review see Refs. 2 and 26). In contrast, reduced muscle activity by hindlimb suspension, immobilization, or functional denervation has opposite effects (27, 28). The dose-dependent effect of CsA treatment on fiber type and MHC expression was mainly restricted to soleus muscle. This is consistent with the observation that the calcineurin content was higher in slow fibers of soleus muscle than in plantaris. Moreover, the nuclear localization of calcineurin in slow fibers, already observed in insulin-like growth factor-1-activated skeletal myocytes (29), reflects the co-translocation of calcineurin with NFAT necessary for the transcriptional activity of this factor (5, 30). One intriguing result was the limited range of the slow to fast MHC transition. MHC expression undergoes the slow to fast transition in the order MHC-1 ⇒ MHC-2A ⇒ MHC-2X ⇒ MHC-2B. Only three SOL samples contained a small percentage of MHC-2X and even MHC-2B isoforms in all SOL samples (15). Because a similar dose of CsA was able to completely inhibit the dephosphorylation of NFAT by calcium in rat spleen cells (31), the fiber type transition induced by calcium inactivating agent (32). In some studies, CsA treatment failed to affect the MHC composition of either slow or fast twitch muscle. This discrepancy could be explained by the higher blood CsA concentrations obtained in our study (7) or by the use of plantaris muscle comprising nearly 90% fast fibers under control conditions (8). Whatever, the present results unambiguously show that even at rest the calcineurin pathway could influence the expression of contractile proteins in a typically slow twitch muscle.

Moreover, a strong co-expression of SERCA1 with fast MHC isoforms and SERCA2a with MHC-1 was found in SOL mus-
cles, in a CsA-dependent manner. Previous studies clearly showed that the pattern of expression of SERCA protein isoforms is under the control of various factors including changes in contractile activity and active loading (2, 12). Our results show, for the first time, that the expression of SERCA and MHC isoforms can be co-regulated by calcineurin.

Metabolic Phenotype—We have studied whether families of proteins involved in a fiber type-specific manner in muscle energy metabolism and whose expression is known to be dependent on muscle activity would respond to calcineurin inhibition in vivo as well. In slow muscle, CsA treatment resulted in an increase in the specific activity of M-LDH, the major isoform in glycolytic muscle, whereas H-LDH expression remained constant. This result is expected from a slow to fast phenotype transition and in this respect mimics the effects of muscle unloading (15). CK is also a multigenic family whose expression, organization, and function varies in a tissue-specific and developmentally regulated manner (33–35). The high shortening speed of fast twitch fibers is associated with high total and MM-CK activity providing an efficient ATP regenerating system for contraction (36). In contrast, oxidative muscles exhibit a high specific activity of mi-CK with lower total CK (37, 38). CK expression was sensitive to calcineurin inhibition with CsA treatment inducing a 36% increase in total and MM-CK activity. Again these results conform to the effects of muscle unloading (15) and reflect a slow to fast phenotype transition of the soleus muscle.

Activities of mi-CK and CS can be considered as markers of muscle oxidative capacity. A significant increase in CS activity was observed in the soleus muscle of treated rats, and both CS and mi-CK activities were positively correlated with blood level of CsA, suggesting a calcineurin-dependent change in oxidative capacities. It is noticeable that none of these markers were affected by vehicle alone. This strongly supports our previous conclusion that the vehicle of CsA induces a mitochondrial poisoning (14) rather than a decreased mitochondrial content. On the other hand, the increased oxidative capacity could at first be an apparent discrepancy with the slow to fast phenotype transition. However, increased mitochondrial content following CsA treatment is entirely consistent with a shift from slow oxidative to fast oxidative fibers, because type IIA fibers are known to have higher oxidative potential than type I fibers in rat (13).

Capillary Supply—As expected from the slow to fast transition induced by the calcineurin pathway inhibition, CsA treatment induced a decrease in C/F in slow but not in fast twitch muscle. Although the morphometric analysis of capillary network has limitations, the most widely used supply indexes are C/F and CD (39). It is interesting in this respect that CsA treatment resulted in capillary regression in slow muscle, suggesting that the calcineurin pathway is also involved in the regulation of capillary growth. However, the decrease in C/F was not sufficient to have profound functional consequences, because the fiber atrophy resulted in an increased CD likely secondary to decreased fiber size in both muscles.

Calcineurin and Muscle Phenotype—In soleus muscle, inhibition of calcineurin by CsA induced phenotype changes that seem to be mostly restricted to the transition from slow oxidative type I to fast oxidative type IIA phenotype. Indeed, whereas type IIA fibers express contractile and sarcoplasmic reticulum proteins of the fast type, these fibers exhibit a higher oxidative and glycolytic potential than slow type I fibers, resulting in resistance to fatigue despite increased contraction rate and energy consumption. We found a CsA-dependent decrease in the expression of slow MHC and SERCA2a and an increase in SERCA1 and fast MHC-2A, compatible with the slow to fast transition. A CsA-dependent increase in mi-CK and CS was also observed, in line with increased oxidative capacity, associated with a CsA-dependent increase in M-CK and M-LDH compatible with the higher glycolytic potential of type IIA fibers. Taken together, these results strongly suggest that the calcineurin pathway is involved in the fast IIA to slow I phenotype transition in soleus muscle. Closely related transcriptional protein-DNA complexes have been demonstrated to confer the fiber type specificity between slow and fast fibers (40). Recently Chin et al. (4) examined the DNA sequence of these fiber type-specific promoter regions and could identify a NFAT binding sequence in the promoter of slow type proteins, which is absent in the fast promoter; this NFAT recognition sequence is present in the troponin I, myoglobin, and mi-CK genes. Because calcineurin inhibition leads to an increase or decrease in specific protein isoforms, the NFAT sequence should participate in up- or down-regulation of these specific genes. The strong correlation between blood CsA and mi-CK activity suggests that the NFAT recognition sequence on the mi-CK gene promotes mi-CK gene down-regulation compatible with fast oxidative toward slow oxidative phenotype transition. Our data suggest a similar regulation for other genes like SERCA1, MHC-2A, M-LDH, and M-CK, whereas MHC-1 and SERCA2a should be up-regulated. Information concerning the NFAT recognition sequences of muscle specific genes and their participation in the regulation of the transcription will be of uppermost interest in the next future for the understanding of muscle adaptation.

In adult skeletal muscle, the slow phenotype results from continuous nerve stimulation and is abolished by denervation of decreased activity, suggesting a basal activation of the calcineurin pathway. The calcineurin-dependent activation of the transcription factor NFAT is enhanced by sustained low amplitude elevation of internal calcium (41) naturally occurring in slow muscle or induced by sustained stimulation of fast muscle. The observation that the catalytic subunit of CaMKIIa is more abundant in slow twitch muscle adds support to the proposal that calcineurin can mediate the effects of nerve activity in this muscle. Moreover, it can be further suggested that the absence of sensitivity of fast muscle to CsA treatment is because of the low expression of CaMKIIa in this muscle. In this respect, our results are in line with those of others (8) who observed an effect of calcineurin inhibition on the plantaris muscle phenotype only following increased activation. This would suggest that the pattern of activity can also govern the expression of calcineurin itself, which will then initiate the fast to slow transition in this muscle. This hypothesis is reinforced by two recent reports. First, it has been shown that CnA is up-regulated in insulin-like growth factor-1-transfected skeletal myocytes (29), and second, transgenic mice expressing a constitutively active calcineurin in skeletal muscle have an increased number of slow fibers in their fast skeletal muscles (32).

Conclusions—We showed that calcineurin inhibition by CsA could induce qualitative and quantitative changes in the expression of multigenic protein families involved in contraction, calcium homeostasis, and energy metabolism, compatible with a slow to fast phenotype transition. The changes in contractile machinery (decreased MHC-1 and increased MHC-2A isoforms), calcium regulation (SERCA1 and SERCA2a isoforms), and metabolic pathway (increased M-CK and M-LDH isoenzymes) observed in this study are compatible with a calcineurin-induced type IIA toward type I phenotype conversion. These results strongly support the proposal that calcium-mediated calcineurin activation is one of the transduction pathways linking sustained contractile activity and the transition between fast oxidative to slow oxidative phenotype in slow muscle.
A number of studies have reported adverse effects of immunosuppressive treatments on exercise capacity and skeletal muscle function (42, 43) of transplanted patients. Slow to fast phenotype transition induced by CsA per se and mitochondrial poisoning by the vehicle (14) can both contribute to skeletal muscle fatigability and decreased endurance performance in these patients.

Acknowledgments—We acknowledge R. Fischmeister for continuous support and E. Boehm for careful reading of the manuscript.

REFERENCES

1. Buller, A. J., Eccles, J. C., and Eccles, R. M. (1960) J. Physiol. (Lond.) 150, 417–439
2. Pette, D., and Vrbova, G. (1999) Muscle Nerve 22, 666–677
3. Buonomano, A., and Rosenthal, N. (1996) Dev. Genet. 19, 95–107
4. Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, W., Bassel-Duby, R., and Williams, R. S. (1998) Genes Dev. 12, 2499–2509
5. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu. Rev. Imunol. 15, 113–116
6. Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Cell 66, 807–815
7. Biring, M. S., Fournier, M., Ross, D. J., and Lewis, M. I. (1998) J. Physiol. 526, 1967–1975
8. Dunn, S. E., Burns, J. L., and Michel, R. N. (1999) J. Biol. Chem. 274, 21908–21912
9. Briggs, F. N., Poland, J. L., and Solaro, R. J. (1977) J. Physiol. (Lond.) 266, 587–594
10. Wu, K. D., Lee, W. S., Wey, J., Bungard, D., and Lytton, J. (1995) Am. J. Physiol. 269, C775–C784
11. Schulte, L. M., Navarro, J., and Kandarian, S. C. (1993) Am. J. Physiol. 264, C1308–C1315
12. Kandarian, S. C., Peters, D. G., Taylor, J. A., and Williams, J. H. (1994) Am. J. Physiol. 266, C1190–C1197
13. Smith, D., Green, H., Thomson, J., and Sharratt, M. (1988) Am. J. Physiol. 254, C661–C668
14. Sanchez, H., Bigard, X., Vekler, V., Mettauer, B., Lampert, E., Lonsdorfer, J., and Ventura-Clapier, R. (2000) J. Mol. Cell. Cardiol. 32, 323–331
15. Bigard, A. X., Boehm, E., Vekler, V., Mateo, P., Anflous, K., and Ventura-Clapier, R. (1998) J. Mol. Cell. Cardiol. 30, 2391–2401
16. Hoppeler, H., and Kayar, S. R. (1988) News Physiol. Sci. 3, 113–116
17. Skorjanc, D., Jaschinski, F., Heine, G., and Pette, D. (1998) Am. J. Physiol. 3810–C818
18. Desplanches, D., Favier, R., Sempero, B., and Hoppler, H. (1991) J. Appl. Physiol. 71, 2419–2424
19. Dubowitz, V. (1985) Muscle Biopsy. A Practical Approach, 2nd Ed., pp. 28–29, Baillière Tindal, London
20. Skorpjanc, D., Jaschinski, F., Heine, G., and Pette, D. (1998) Am. J. Physiol. 3810–C818
21. Schiaffino, S., and Reggiani, C. (1996) Physiol. Rev. 76, 371–422
22. Schiaffino, S., and Reggiani, C. (1996) Physiol. Rev. 76, 371–422
23. Schiaffino, S., and Reggiani, C. (1996) Physiol. Rev. 76, 371–422
24. Molkentin, J. D., Lu, J.-R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215–228
25. Ho, I. C., Kim, J. H., Tooney, J. W., Spegelman, B. M., and Glimecher, L. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15537–15541
26. Schiaffino, S., and Reggiani, C. (1996) Physiol. Rev. 76, 371–422
27. Thomason, D. B., and Booth, F. W. (1990) J. Appl. Physiol. 68, 1–12
28. Booth, F. W. (1982) J. Appl. Physiol. 52, 1113–1118
29. Musaro, A., McCallagh, K. J. A., Naya, F. J., Olson, E. N., and Rosenthal, N. (1999) Nature 400, 581–585
30. Zhu, J., and McKeon, F. (1999) Nature 398, 256–260
31. Mende, U., Kagen, A., Cohen, A., Aramburu, J., Schoen, F. J., and Neer, E. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13893–13898
32. Naya, F. J., Mercer, B., Shelton, J., Richardson, J. A., Williams, R. S., and Olson, E. N. (2000) J. Biol. Chem. 275, 4545–4548
33. Wegmann, G., Zanolla, E., Eppenberger, H. M., and Wallimann, T. (1992) J. Muscle Res. Cell Motil. 13, 420–435
34. Ventura-Clapier, R., Kuznetsov, A., Vekler, V., Boehm, E., and Anflous, K. (1998) Mol. Cell. Biochem. 184, 231–247
35. Payne, R. M., and Strauss, A. W. (1994) Mol. Cell. Biochem. 133, 235–243
36. Watchko, J. F., Daood, M. J., and La Bella, J. J. (1990) Pediatr. Res. 27, 415–422
37. Saks, V. A., Khuchua, Z. A., Vasilyeva, E. V., Belikova, O. Y., and Kuznetsov, A. V. (1994) Mol. Cell. Biochem. 133, 155–192
38. Qin, W. N., Khuchua, Z., Boero, J., Payne, R. M., and Strauss, A. W. (1999) Mol. Cell. Biochem. 215–228
39. Hudlicka, O., Brown, M., and Eginton, S. (1992) Physiol. Rev. 72, 369–417
40. Nakayama, M., Stauffer, J., Cheng, J., Banerjee-Basu, S., Wawrousek, E., and Bluemen, A. (1996) Mol. Cell. Biol. 16, 2408–2417
41. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998) Nature 398, 933–936
42. Mettauer, B., Lampert, E., Petitjean, P., Bogui, P., Epailly, E., Schnedelecker, B., Geny, B. E., Haberey, P., and Lonsdorfer, J. (1996) Int. J. Sports Med. 17, 277–286
43. Kao, A. C., Van Trigt, P., Shaffer-McCall, G. S., Shaw, J. P., Kuzil, B. B., Page, R. D., and Higginbotham, M. B. (1994) Circulation 89, 2605–2615
Calcineurin Co-regulates Contractile and Metabolic Components of Slow Muscle Phenotype
Xavier Bigard, Hervé Sanchez, Joffrey Zoll, Phillipe Mateo, Vincent Rousseau, Vladimir Veksler and Renée Ventura-Clapier

J. Biol. Chem. 2000, 275:19653-19660.
doi: 10.1074/jbc.M000430200 originally published online April 20, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000430200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 7 of which can be accessed free at http://www.jbc.org/content/275/26/19653.full.html#ref-list-1