Am J Physiol Regul Integr Comp Physiol 294: R1319–R1328, 2008.
First published January 23, 2008; doi:10.1152/ajpregu.00631.2007.

Adaptive responses to creatine loading and exercise in fast-twitch rat skeletal muscle

Maria Gallo,[1] Ian MacLean,[1] Neil Tyreman,[2,3] Karen J. B. Martins,[1] Daniel Syrotuik,[1] Tessa Gordon,[2,3] and Charles T. Putman[1,3]

[1]*Exercise Biochemistry Laboratory, Faculty of Physical Education and Recreation, and* [2]*Division of Physical Medicine and Rehabilitation and* [3]*Centre for Neuroscience, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada*

Submitted 31 August 2007; accepted in final form 22 January 2008

Gallo M, MacLean I, Tyreman N, Martins KJ, Syrotuik D, Gordon T, Putman CT. Adaptive responses to creatine loading and exercise in fast-twitch rat skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 294: R1319–R1328, 2008. First published January 23, 2008; doi:10.1152/ajpregu.00631.2007.—We investigated the effects of chronic creatine loading and voluntary running (Run) on muscle fiber types, proteins that regulate intracellular Ca^{2+}, and the metabolic profile in rat plantaris muscle to ascertain the bases for our previous observations that creatine loading results in a higher proportion of myosin heavy chain (MHC) IIb, without corresponding changes in contractile properties. Forty Sprague-Dawley rats were assigned to one of four groups: creatine-fed sedentary, creatine-fed run-trained, control-fed sedentary, and control-fed run-trained animals. Proportion and cross-sectional area increased 10% and 15% in type IIb fibers and the proportion of type IIa fibers decreased 11% in the creatine-fed run-trained compared with the control-fed run-trained group ($P < 0.03$). No differences were observed in fast Ca^{2+}-ATPase isoform SERCA1 content ($P > 0.49$). Creatine feeding alone induced a 41% increase ($P < 0.03$) in slow Ca^{2+}-ATPase (SERCA2) content, which was further elevated by 33% with running ($P < 0.02$). Run training alone reduced parvalbumin content by 50% ($P < 0.05$). By comparison, parvalbumin content was dramatically decreased by 75% ($P < 0.01$) by creatine feeding alone but was not further reduced by run training. These adaptive changes indicate that elevating the capacity for high-energy phosphate shuttling, through creatine loading, alleviates the need for intracellular Ca^{2+} buffering by parvalbumin and increases the efficiency of Ca^{2+} uptake by SERCAs. Citrate synthase and 3-hydroxyacyl-CoA dehydrogenase activities were elevated by run training ($P < 0.003$) but not by run training + creatine feeding. This indicates that creatine loading during run training supports a faster muscle phenotype that is adequately supported by the existing glycolytic potential, without changes in the capacity for terminal substrate oxidation.

fiber type transitions; myosin heavy chain; Ca^{2+}-ATPase; SERCA; parvalbumin

IN RODENT SKELETAL MUSCLES, exercise training leads to structural, contractile, and metabolic changes that enhance fatigue resistance and improve the efficiency of muscle contraction. Importantly, these molecular and physiological adaptations encompass the transformation of contractile proteins from fast to slower isoforms (1, 12, 30, 46, 62) and increase oxidative capacity (20, 30, 33, 55). Endurance exercise training of rodent muscles has been shown to induce myosin heavy chain (MHC)-based fiber type transitions typified by increases in type I and IIa fibers and corresponding decreases in the fastest type IId/x and IIb fibers (20, 45). In a previous study (16), we showed that muscle creatine loading attenuated running-induced fast-to-slow MHC isoform transitions as determined by SDS-PAGE. Specifically, creatine loading prevented increases in MHC IIa and induced an increase in MHC IIb at the expense of MHC IId/x. Without detailed immunohistochemical analyses, however, it was not possible to discern whether changes in the proportions of the various MHC isoforms resulted from fiber type transitions within the fast-twitch type II subpopulations or from fiber-specific hypertrophy or atrophy.

In our previous study (16), we did not detect reductions in time to peak twitch tension, half-rise time, or half-fall time, despite a significant transition toward a faster muscle fiber phenotype. Expression levels of intracellular proteins that regulate Ca^{2+} kinetics (i.e., Ca^{2+}-ATPase isoforms and parvalbumin) can alter the properties of the excitation-contraction-relaxation cycle and could explain the apparent disconnect between contractile properties and muscle fiber phenotype. Green et al. (19) reported substantial reductions in the content of the Ca^{2+}-buffering protein parvalbumin in rat fast-twitch muscle that paralleled the transformation of muscle fibers from a fast-fatigable to a slow-oxidative phenotype after 15 wk of endurance training. Using another model of endurance training (e.g., chronic low-frequency stimulation), other investigators (10, 22, 26, 31, 37) reported similar reductions in parvalbumin content in several rodent models that also corresponded to fast-to-slow fiber type transitions and were found to correlate with prolongation of the excitation-contraction-relaxation cycle (10, 60). These (10, 19, 22, 26, 31, 37) and other related studies (23, 24, 31, 35, 36) also consistently showed activity-induced decreases in the fast Ca^{2+}-ATPase isoform [sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA1)] and increases in the slow SERCA2 isoform within rodent fast-twitch muscles.

Exercise-induced fast-to-slow fiber type transitions in the rat are also accompanied by a shift from those that are primarily reliant on glycogenolytic ATP production to those that rely on aerobic pathways of energy production (19, 20, 33). Maximal citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HADH) activities have been shown to be proportional to the maximum aerobic capacity, whereas phosphofructokinase (PFK) and GAPDH activities consistently reflect glycolytic potential (45, 47).

The purpose of the present study was to determine whether enhancing the cellular energy status of the plantaris muscle by

Address for reprint requests and other correspondence: C. T. Putman, E-417 Van Vliet Centre, Univ. of Alberta, Edmonton, AB, Canada T6G 2H9 (e-mail: tputman@ualberta.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

chronic creatine feeding would allow this fast-twitch muscle to retain its fast-glycolytic phenotype after prolonged voluntary running. A secondary purpose was to investigate changes in the proteins that are known to regulate intracellular Ca^{2+} kinetics to explain the apparent disconnect between muscle fiber types and isometric functional measures.

METHODS

Animals and care. Forty male weanling (3-wk-old) Sprague-Dawley rats, which were part of a previous related study (16), were examined in the present study. All experiments were completed in accordance with the guidelines of the Canadian Council for Animal Care and received ethical approval from the University of Alberta Health Sciences Animal Welfare and Policy Committee. Animals were individually housed under controlled environmental conditions (22°C and 12:12-h light-dark cycle) and ingested high-protein rat chow and their assigned drinking solutions ad libitum. Body mass and food and solution intake were monitored throughout the study and are reported elsewhere (16). Total creatine content of the plantaris muscles was elevated by 22% after the animals consumed a solution of 1% creatine and 5% dextrose ad libitum for 13 wk, also as previously reported (16).

Experimental design. A 13-wk endurance running protocol on activity wheels (Wahmann, Baltimore, MD) was carried out according to a procedure described by Morse et al. (41) and Gallo et al. (16). Animals were randomly assigned to one of the following groups (n = 10 per group): creatine-fed sedentary (Cre-Sed), creatine-fed run-trained (Cre-Run), control-fed sedentary (Con-Sed), and control-fed run-trained (Con-Run). The run-trained groups had access to the activity wheels for 12 h/day. There were no differences ($P > 0.64$) between Cre-Run and Con-Run groups with regard to daily or total distance run throughout the study (16). The creatine-fed groups consumed a 1% creatine solution (Createam, NutraSense, Shawnee Mission, KS) in 5% dextrose (Fisher Scientific, Fairlawn, NJ); the control groups consumed a 5% dextrose solution.

Muscle sampling. On completion of the study, muscles were collected from heavily anesthetized (45 mg/kg ip pentobarbital sodium; MTC Pharmaceutical, Cambridge, ON, Canada) animals according to Gallo et al. (17). Plantaris muscles that were used for immunohistochemical analyses were isolated from the left hindlimb, weighed, fixed in a slightly stretched position, and frozen in melting isopentane (−156°C). Plantaris muscles from the right hindlimbs were freeze clamped using clamps that were prechilled in liquid N_2, stored in liquid N_2, and subsequently used for biochemical analyses. Animals were euthanized with an overdose of pentobarbital sodium (100 mg/kg) and then exsanguinated.

Antibodies for immunohistochemistry. Monoclonal antibodies directed against adult MHC isoforms (58, 59) were harvested from hybridoma cell lines (American Type Culture Collection, Manassas, VA): BA-D5 (IgG and anti-MHC I), SC-71 (IgG and anti-MHC IIa), BF-F3 (IgM and anti-MHC IIb), and BF-35 (IgG and all MHCs, but not MHC IId/x). Biotinylated horse anti-mouse IgG (rat-absorbed, affinity-purified), biotinylated horse anti-goat IgG, and biotinylated goat anti-mouse IgM were obtained from Vector Laboratories (Burlingame, CA), and nonspecific control mouse IgG was obtained from Santa Cruz Biochemicals (Santa Cruz, CA).

Immunohistochemistry for myosin. Plantaris muscles were mounted in embedding medium (Tissue-Tek OCT Compound, Miles Scientific), and 10-μm-thick frozen sections were collected from the midpoint of each muscle at −20°C. Immunocytochemical staining was completed according to Putman et al. (52). Sections were air-dried, washed in PBS with 0.1% Tween 20 (PBS-T) and then with PBS, and incubated for 15 min in 3% H_2O_2 in methanol. Serial sections stained for MHC I, MHC IIa, and all MHCs, but not MHC IId/x, were incubated at room temperature for 1 h in a blocking solution [*blocking solution 1*: 1% BSA and 10% horse serum in PBS-T (pH 7.4)] containing Avidin-D Blocking Reagent (Vector Laboratories); on sections stained for MHC IIb, goat serum was substituted for horse serum (*blocking solution 2*). Sections were incubated overnight at 4°C with the primary antibody diluted in its corresponding blocking solution, which contained a biotin blocking reagent (Vector Laboratories). Antibodies were diluted as follows: BA-D5 at 1:400, SC-71 at 1:100, BF-35 at 1:10,000, and BF-F3 at 1:400. Sections were washed as described above and incubated for 1 h with biotinylated horse anti-mouse IgG (1:200 dilution; MHC I and MHC IIa, but not MHC IId/x) or biotinylated goat anti-mouse IgM (1:400 dilution; MHC IIb). Sections were washed and incubated with Vectastain ABC Reagent [i.e., avidin-biotin-horseradish peroxidase (HRP) complex, Vector Laboratories], and immunoreactivity was developed by incubation with a solution containing diaminobenzidine, H_2O_2, and $NiCl_2$ in 50 mM Tris·HCl (pH 7.5; Vector Laboratories). Control samples were run in parallel; in these samples, the primary IgM antibody was omitted, or a nonspecific mouse IgG antibody was substituted (Santa Cruz Biochemicals). All sections were subsequently dehydrated, cleared, and mounted in Entellan (Merck, Darmstadt, Germany).

Immunohistochemical analyses. All semiquantitative analyses were completed with a Leitz Diaplan microscope (Enrst Leitz Wetzlar) fitted with a Pro Series high-performance charge-coupled device camera (Media Cybernetics) and a custom-designed analytical imaging program (51). Muscle fibers stained for the various MHC isoforms from three distinct cross-sectional areas (CSAs) of the plantaris muscle (i.e., deep, middle, and superficial) were examined for each of the Cre-Sed (185 ± 18 total fibers/muscle), Cre-Run (210 ± 16 total fibers/muscle), Con-Sed (190 ± 14 total fibers/muscle), and Con-Run (198 ± 19 total fibers/muscle) groups. A total of 7,830 fibers were examined for fiber type distribution analyses. Fiber area analyses were performed on the same fibers. Type I, IIa, and IIb fibers were identified by positive staining, and type IId/x fibers were identified by the absence of staining with clone BF-35, as well as all other antibodies. An individual who was blind to the treatment conditions of each muscle completed all histological analyses.

Parvalbumin Western blot analyses. Frozen plantaris muscles were pulverized under liquid N_2. An aliquot of muscle powder was diluted 1:4 in a buffer [20 mM Tris·HCl, 300 mM sucrose, and 0.2 mM PMSF (pH 7.4)] and homogenized using a glass homogenizer (Kontes Glass, Vineland, NJ). Samples were stirred on ice for 20 min and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was collected and stored at −20°C. Protein concentrations were determined according to Bradford (8). Samples were diluted to a final concentration of 1 μg/μl with the homogenization buffer containing 0.1% (wt/vol) bromphenol blue and heated for 10 min at 65°C. Electrophoresis was performed on a 1.5-mm-thick 15% (wt/vol) polyacrylamide minigel (3.5% stacking gel; Protean-II, Bio-Rad Laboratories, Mississauga, ON, Canada) at 25–40 mA for 2 h (Mini Trans Blot Cell, Bio-Rad Laboratories). Equal amounts of protein (10 μg/lane) were loaded. Proteins were electrotransferred (wet) onto a polyvinylidene difluoride membrane (63) and stained with Ponceau S (Sigma-Aldrich, Oakville, ON, Canada) as confirmation of equal loading between lanes. Membranes were destained, blocked in a buffer containing skim milk powder (5% wt/vol) and PBS-T (0.1% vol/vol) for 1 h, and incubated for 1 h with rabbit polyclonal anti-parvalbumin (0.1 μg/ml in the blocking solution, IgG; Abcam, Cambridge, MA). Membranes were then washed and incubated for 1 h with anti-rabbit IgG-HRP (1:3,000 in the blocking solution; Vector Laboratories) and washed several times. Immunoreactivity was visualized with detection reagents (Amersham Biosciences, Montreal, QC, Canada) and corresponded to that of a 12-kDa molecule, as determined by comparison with standard molecular weight markers (Precision Plus Protein Standards, Bio-Rad Laboratories). All samples were evaluated in duplicate. Immunoblots were evaluated by integrating densitometry using GeneSnap and GeneTools (Chemigenius Gel Documentation System, Syngene). Membranes were reprobed with monoclonal anti-α-actinin (diluted 1:500 in blocking solution; clone

EA-53, Sigma-Aldrich), which served as the internal control and further confirmed equal loading between lanes. Immunoreactivity for α-actinin (100 kDa) was visualized as described above after incubation with anti-mouse IgG (diluted 1:2,000 in blocking solution; Vector Laboratories).

Ca^{2+}-ATPase Western blot analyses. Frozen plantaris muscle samples were minced and homogenized using a glass homogenizer in a buffer (diluted 1:4) containing 50 mM Tris·HCl, 1 mM Na_2EDTA, 0.1% Triton X-100, and 5 mg/ml of protease inhibitor (pH 7.6). Homogenates were stirred on ice for 20 min and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was collected and stored at −20°C. Protein concentrations were determined as described above. Samples were further diluted as described above and heated for 3 min at 85°C. Equal amounts of protein were loaded (50 and 80 μg/lane for SERCA1 and SERCA2, respectively). Electrophoresis was performed on 1.5-mm-thick 7% (wt/vol) polyacrylamide minigels (3.5% stacking gel; Protean-II, Bio-Rad Laboratories) for 30 min at 60 V and, subsequently, for 90 min at 140 V. Separated proteins were transferred to a polyvinylidene difluoride membrane (63). Equal loading was confirmed using Ponceau S (see above). Membranes probed for Ca^{2+}-ATPase isoforms were blocked in a buffer containing skim milk powder (2.5% wt/vol) and BSA (1% wt/vol) in PBS-T (0.1%, pH 7.4) and then incubated with monoclonal anti-SERCA1 (diluted 1:5,000 in blocking solution; Abcam) or monoclonal anti-SERCA2 (1:4,000 dilution; Abcam) overnight at 4°C. Membranes probed for SERCA1 were washed and incubated with anti-mouse IgG-HRP (diluted 1:3,000 in blocking solution; Vector Laboratories) for 1 h, washed again, and developed as described above. Membranes probed for SERCA2 were incubated with biotinylated anti-mouse IgG (diluted 1:400 in blocking solution; Vector Laboratories) for 1 h and incubated again for 1 h with peroxidase-labeled streptavidin (diluted 1:500 in blocking solution; KPL, Gaithersburg, MD), washed, and developed as described above. Immunoreactivity for both Ca^{2+}-ATPase isoforms corresponded to that of a 110-kDa molecule. All samples were evaluated in duplicate (see above). Membranes were reprobed with polyclonal anti-β-actin (1:2,000 dilution; Abcam), which served as the internal control and further confirmed equal loading. Immunoreactivity of β-actin was

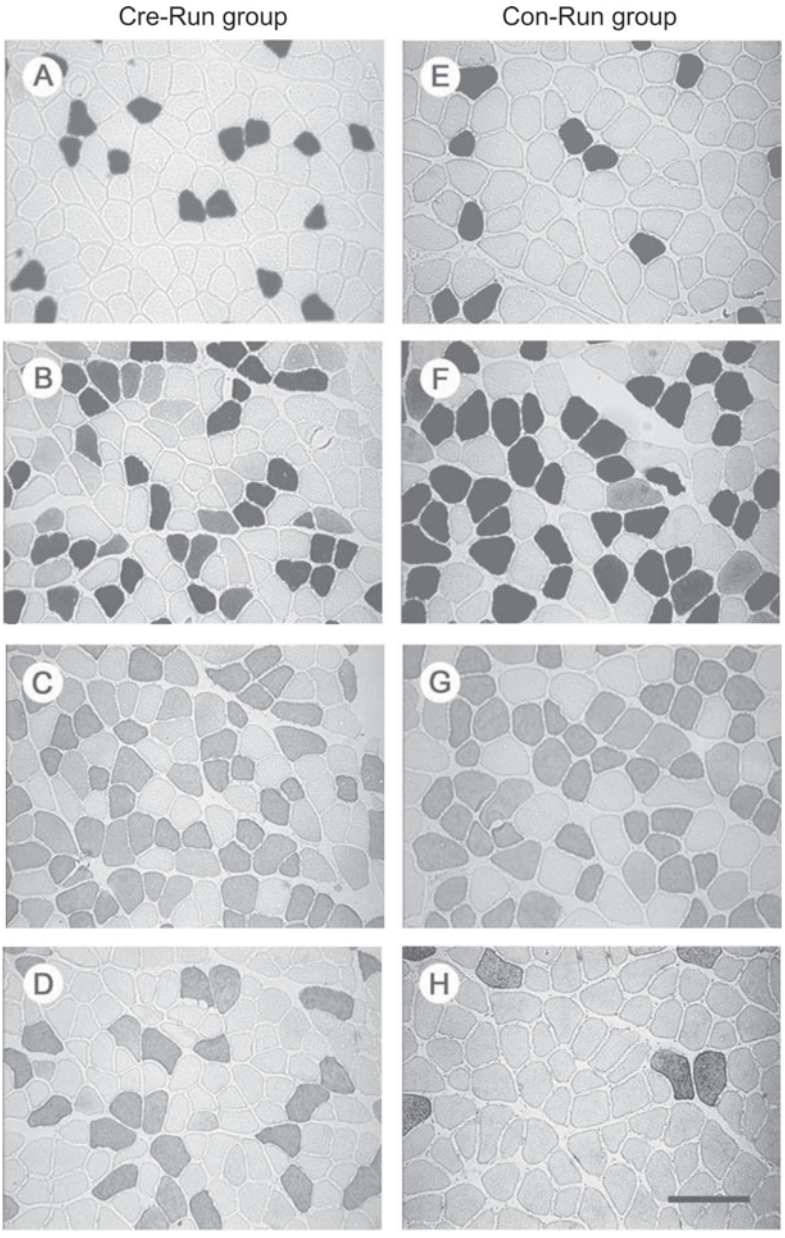

Fig. 1. Representative photomicrographs of myosin heavy chain (MHC) immunohistochemistry of plantaris muscle from creatine-fed run-trained (Cre-Run, *A–D*) and control-fed run-trained (Con-Run, *E–H*) groups. *A* and *E*: immunostains of MHC I (clone BA-D5); *B* and *F*: immunostains of MHC IIa (clone SC-71); *C* and *G*: immunostains of all MHC isoforms except MHC IId/(x) (clone BF-35); *D* and *H*: immunostains of MHC IIb (clone BF-F3). Scale bar, 100 μm.

visualized as described above after incubation with a biotinylated anti-rabbit IgG (diluted 1:2,000 in blocking solution; Vector Laboratories) and peroxidase-labeled streptavidin (diluted 1:500 in blocking solution; KPL).

Metabolic enzyme measurements. The tricarboxylic acid cycle (CS and HADH) and glycolytic (GAPDH and PFK) reference enzymes examined in this study are equilibrium enzymes that display zero kinetics in vivo; thus their maximum activity is directly proportional to their respective protein contents (45, 47). For measurements of CS (EC 4.1.3.7), HADH (EC 1.1.1.35), GAPDH (EC 1.2.1.12), and PFK (EC 2.7.1.11), muscles were extracted in a high-salt medium containing 5 mM EDTA and 100 mM sodium/potassium phosphate buffer (pH 7.2) (52), with the addition of 0.1% (vol/vol) Triton X-100, to ensure complete extraction of soluble and structure-bound activities. To stabilize GAPDH, DTT was added to an aliquot of the supernatant fraction yielding a 2 mM final concentration. Similarly, for stabilization of PFK, fructose 1,6-bisphosphate and DTT were added to an aliquot of the supernatant fraction yielding final concentrations of 1 and 2 mM, respectively. Maximal CS activity was subsequently measured at 30°C (52, 61). Maximal HADH activity was also determined at 30°C as described elsewhere (3, 52). Maximal PFK and GAPDH activities were immediately measured after homogenization at 30°C (3, 52).

Statistical analyses. Data are normally distributed and summarized as means ± SE. Differences between group means were assessed using a two-way analysis of variance. When a significant F ratio was found, differences were located using the least significant difference post hoc analysis for planned comparisons. Differences were considered significant at $P < 0.05$, but actual P values are cited.

RESULTS

Fiber type transitions. Fiber type transitions of the plantaris muscle were assessed by semiquantitative immunohistochemical analyses on serial sections (Fig. 1) in the deep, middle, and superficial regions of each group to ensure representative sampling. As shown in Fig. 2, running increased the proportion of fibers expressing MHC IIa in creatine-fed (by 9%, $P < 0.04$) and control-fed (by 26%, $P < 0.0003$) groups; however, the increase was substantially lower in the creatine group ($P < 0.02$), indicating that the running-induced fiber type transitions were attenuated in the presence of creatine loading. This was reinforced by the finding that the proportion of fibers expressing MHC IIb decreased by 15% ($P < 0.004$) in the Con-Run group compared with the Cre-Run group, which did not differ from its sedentary control. The proportion of fibers expressing MHC IId/x was decreased to the same extent (by ~11%) in the Cre-Run ($P < 0.03$) and Con-Run ($P < 0.05$) compared with their corresponding sedentary controls. No changes were observed in the percentage of fibers expressing MHC I or the proportions of the various hybrid fiber types.

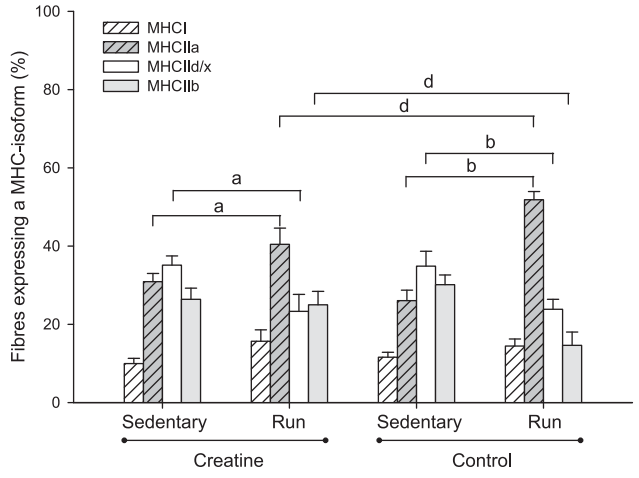

Fig. 2. Percentage of fibers expressing a particular MHC isoform in plantaris muscle. [a]Significant difference between Cre-Run and creatine-fed sedentary (Cre-Sed). [b]Significant difference between Con-Run and control sedentary (Con-Sed). [d]Significant difference between Cre-Run and Con-Run.

CSA. Figure 3 summarizes the CSA of all pure and hybrid fiber types. Running alone was associated with an increase in the CSA of all pure fiber types (Con-Sed vs. Con-Run) and hybrid type IIa/IId(x) fibers. The effects of creatine varied in response to the activity conditions. In the sedentary groups, creatine loading alone was associated with 22%, 39%, 17%, and 21% increases in CSA of type I, I/IIa, IId(x), and IIb fibers, respectively ($P < 0.002$). In the running groups, however, this trend was reversed. Creatine loading combined with running was associated with 29%, 17%, and 9% decreases in the CSA of type IIa/IId(x), IId(x), and IIb fibers, respectively ($P < 0.002$). This resulted from a more advanced fiber type transition from IIb to IIa in the Con-Run (Fig. 4*A*) than the Cre-Run (Fig. 4*B*) group.

Parvalbumin content. Figure 5*A* illustrates the immunoblot method used to quantify parvalbumin protein content. Parvalbumin content was highest in the Con-Sed group (Fig. 5*B*, $P < 0.03$). Running resulted in a ~50% decrease (Con-Run vs. Con-Sed, $P < 0.03$) in parvalbumin content, which corresponded to the fast-to-slower fiber type transitions observed within the subpopulations of fast-twitch fiber types. We were surprised to find that creatine feeding alone resulted in a 75% decrease in parvalbumin content (Con-Sed vs. Cre-Sed, $P < 0.0004$), whereas creatine loading plus running resulted in a similarly low level of parvalbumin, which was only 17% of that in the Con-Run group.

Ca^{2+}-ATPase (SERCA1 and SERCA2) content. The immunoblot methods used to compare the relative abundance of the Ca^{2+}-ATPase isoforms in the plantaris muscle are illustrated in Figs. 6*A* and 7*A*. No significant differences were observed in fast Ca^{2+}-ATPase isoform SERCA1 content (Fig. 6*B*). As expected, running alone resulted in a 43% increase in the slow Ca^{2+}-ATPase SERCA2 (Con-Sed vs. Con-Run, $P < 0.005$). We were surprised to find that SERCA2 content was elevated by 41% (Con-Sed vs. Cre-Sed, $P < 0.03$) in response to creatine loading alone (Fig. 7*B*) and was further elevated by another 33% when creatine loading was combined with running (Cre-Sed vs. Cre-Run, $P < 0.02$).

Reference enzyme activities. All reference enzyme activities are summarized in Fig. 8. Enzyme activities in the plantaris muscle are within ranges previously reported (2, 52). As expected, running alone increased CS (Fig. 8*A*; $P < 0.0001$) and HADH (Fig. 8*B*; $P < 0.05$) activities by 41% and 20%, respectively. In contrast, when creatine loading was combined with running, CS and HADH activities did not increase (Fig. 8, *A* and *B*). No significant differences were observed in the activities of the glycolytic reference enzymes GAPDH ($P = 0.75$) and PFK ($P = 0.77$; Fig. 8, *C* and *D*).

DISCUSSION

Since it was first reported by Harris et al. (25) that oral creatine consumption could elevate total intramuscular creatine

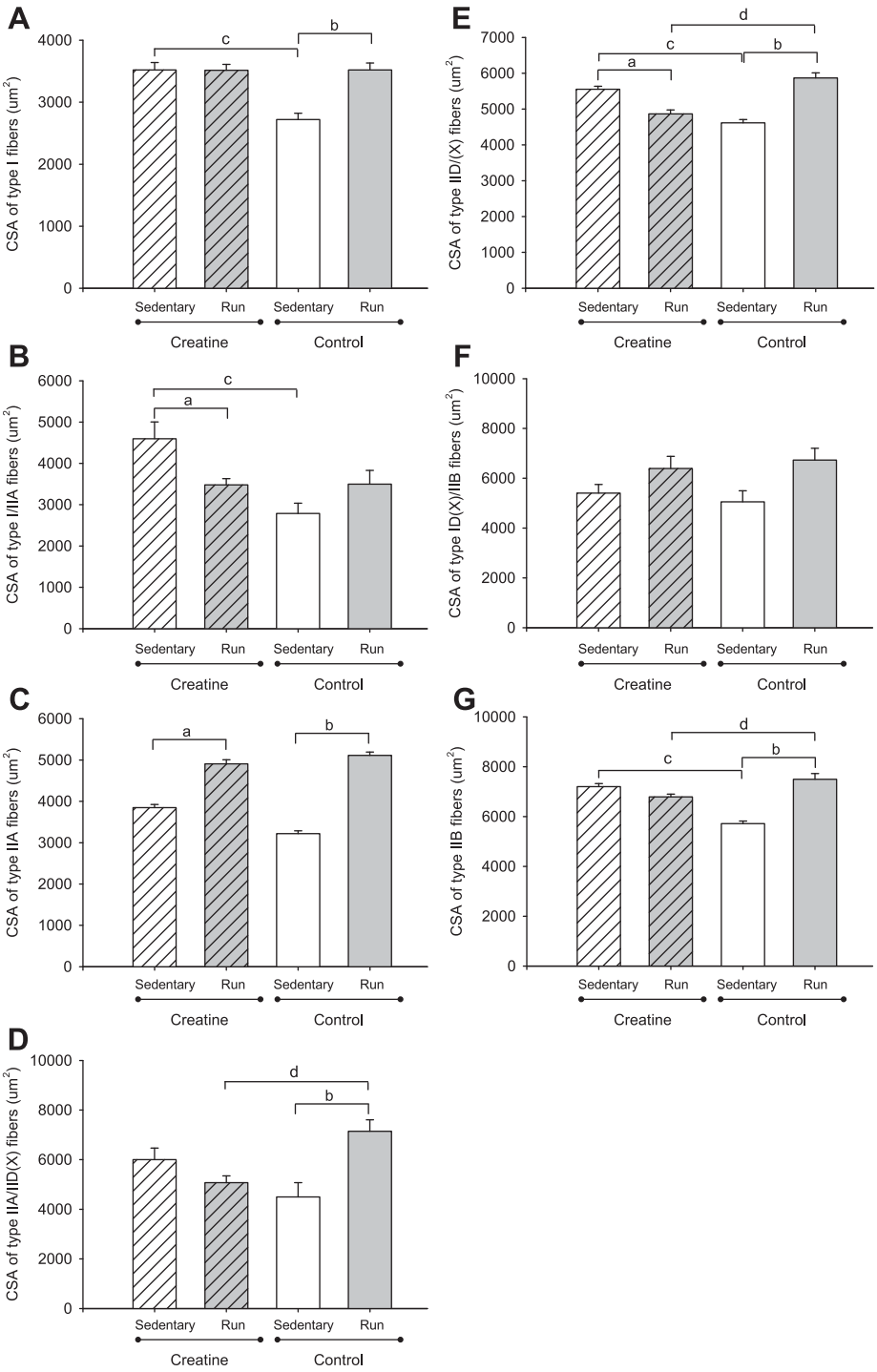

Fig. 3. Cross-sectional area (CSA) of type I (*A*), I/IIa (*B*), IIa (*C*), IIa/IId(x) (*D*), IId(x) (*E*), IId(x)/IIb (*F*), and IIb (*G*) fibers of plantaris muscle. [a]Significant difference between Cre-Run and Cre-Sed. [b]Significant difference between Con-Run and Con-Sed. [c]Significant difference between Cre-Sed and Con-Sed. [d]Significant difference between Cre-Run and Con-Run.

content within human skeletal muscle, the primary focus of subsequent studies has been the extent to which creatine loading acutely improves fatigue resistance by enhancing the available pool of high-energy phosphates (27). In vitro (28) and in vivo (9, 57) studies further reveal the potential for selective myosin synthesis and muscle fiber hypertrophy with prolonged creatine loading. The present study extends the findings of those studies and of our previous work (16), which investigated the effects of voluntary run training and creatine loading on MHC isoform protein contents, whole muscle isometric functional properties, and high-energy phosphates within the plantaris muscle, to include detailed morphological analyses of muscle fiber type distribution and adaptive changes to proteins known to regulate intracellular Ca^{2+} levels and to the metabolic phenotype. Here we report the novel findings that chronic creatine loading in the plantaris muscle attenuated running-induced fast-to-slow fiber type transitions as determined immunohistochemically and prevented associated increases in aerobic capacity as reflected by lower activities of the reference enzymes CS and HADH. Moreover, we were

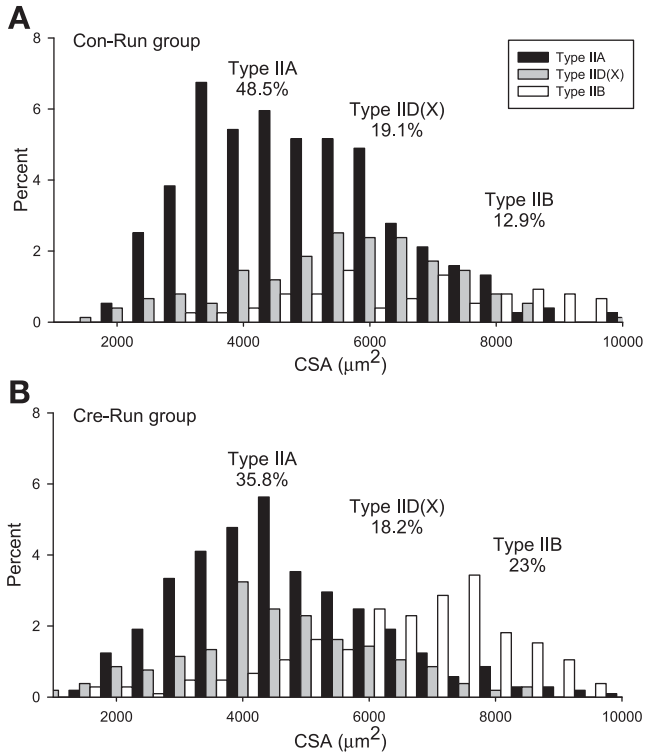

Fig. 4. Myofiber size distribution of type IIa, IId(x), and IIb fibers of plantaris muscle of Con-Run (*A*) and Cre-Run (*B*) groups. Numerical values represent mean proportion of each fiber type.

surprised to find that chronic creatine loading resulted in a dramatic decrease in parvalbumin content and an increase in the slow Ca^{2+}-ATPase isoform SERCA2.

Fiber type transitions. The activity pattern of rats on voluntary running wheels is characterized by many short (i.e., <3 min) bouts of high-intensity (i.e., 40–60 m/min) running corresponding to 95–105% of maximal O_2 consumption (55). Our previous study showed that this training regimen induced fast-to-slow MHC isoform transitions as determined by gel electrophoresis (16) that are qualitatively similar to those found in other forms of endurance exercise training (45, 48). The immunohistochemically determined fiber types in the present study are consistent with the MHC-based isoform transitions seen in our previous study (16). Importantly, the immunohistochemical analyses allowed us to demarcate pure and hybrid fiber types and to further conclude that running-induced MHC isoform transitions were the result of fast-to-slower fiber type transitions within the fast subpopulations and not fiber-specific hypertrophy or atrophy. In contrast, although higher levels of intramuscular creatine prevented the respective decreases and increases in the proportions of type IIb and IIa fibers in response to running, greater MHC IIb content (16) also resulted from an increase in the CSA of type IIb fibers.

Although the underlying mechanisms that account for our observations remain to be elucidated, there is considerable evidence of benefit from an enhanced rate and capacity for high-energy phosphate shuttling during contractile activity in muscles that experience prolonged increases in total creatine content (4, 65). This is supported by recent studies that showed more rapid restoration of the ATP-to-free ADP ratio during recovery from contractile activity by creatine loading (14, 21). In contrast to the fast phenotype supported by creatine loading, muscle creatine depletion by the creatine antagonist β-guanidinoproprionic acid (β-GPA) disrupts high-energy phosphate shuttling (15) and results in muscle fiber atrophy (64) and transformation to a slow oxidative phenotype (15, 44, 54, 56). The changes induced in the present study by creatine feeding and the β-GPA-induced changes reported by others appear to act through alterations in intracellular Ca^{2+} concentration.

In our previous study (16), creatine feeding alone did not alter contraction speed (time to peak twitch tension, half-rise time, or half-fall time) or relaxation (half-fall time) in the plantaris muscle, although it maintained a faster muscle fiber phenotype. This indicates an apparent disconnect between the MHC-based fiber phenotype and the corresponding contractile properties that appears to be primarily related to the adaptive changes within proteins known to regulate intracellular Ca^{2+} (see below). Other factors that influence contractile properties independent of MHC and account for slower contraction speed may include variations in the isoforms of troponin T or myosin light chain (MLC). Leeuw and Pette (38, 39) reported that troponin T isoforms and myosin light chain (MLC) isoforms could exist in atypical combinations within stimulated fast-twitch rabbit muscles and influence contractile properties. For example, earlier onset of the fast-to-slow transition of the regulatory light chains and delayed fast-to-slow exchange of the alkali light chains can generate a spectrum of hybrid combinations. Additionally, a reduction in the MLC3f-to-MLC2f ratio could also lead to decreased speeds of contraction (6, 7, 40) and account for our observations.

Ca^{2+} regulatory proteins. To our knowledge, we are the first to report the effects of creatine loading on the levels of protein

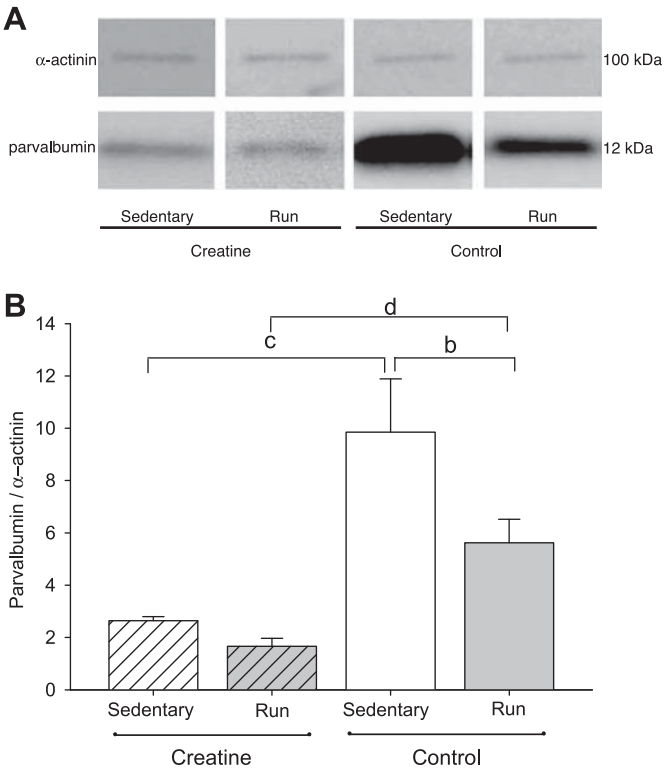

Fig. 5. *A*: representative immunoblot of parvalbumin in plantaris muscle. *B*: densitometric evaluation of *A* as ratio of parvalbumin to α-actinin (loading control). See Fig. 3 legend for significance.

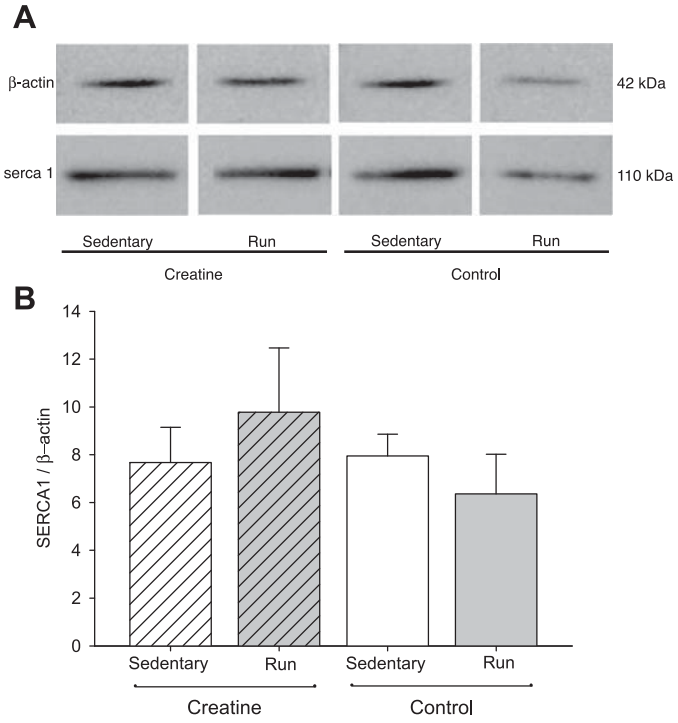

Fig. 6. *A*: representative immunoblot of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA1) in plantaris muscle. *B*: densitometric evaluation of *A* as ratio of SERCA1 to β-actin (loading control).

expression involved in intracellular Ca^{2+} regulation. Previous studies showed that the pattern of SERCA isoform expression is under the control of various factors, including changes in contractile activity (48) and active loading (29). Using cyclosporin A, a calcineurin inhibitor, Bigard and colleagues (5) showed that the expression of SERCA and MHC isoforms can be coregulated by calcineurin and that a strong coexpression of SERCA1 with fast MHC isoforms and SERCA2a with MHC I was found in the soleus, but not plantaris, muscle. An explanation for this disparity may be related to the idea that distinct subsets of MHC-typed fibers are differentially sensitive to neural activation cues mediating the cellular expression of these proteins (13). On the other hand, Zador et al. (66, 67) showed that overexpression of the calcineurin inhibitor CAIN or partial tenotomy prevented the expression of MHC I in regenerating soleus muscle, whereas slow SERCA2 expression remained elevated. These findings reveal that the regulation of SERCA2 expression is distinct from that of the slow myosin and that it may be modulated by neuronal activity but is not entirely dependent on it.

The present study also shows similar asynchronous changes in the proportion of type I fibers and SERCA2 content. Running alone did not increase the proportion of type I fibers, but SERCA2 was significantly increased by 60% (Fig. 7); however, this did correspond to a substantial increase in type IIa fibers. Creatine feeding and running further elevated slow SERCA2 content, whereas the fast-to-slow fiber type transition was attenuated. Interestingly, although creatine feeding alone did not alter the pattern of fiber types, SERCA2 content was remarkably elevated. Thus our data also clearly show that SERCA2 expression is not tightly linked to slow fiber types. The elevation of SERCA2 in response to creatine feeding alone may be related to increases in antiapoptotic proteins, such as Bcl-2, which have been shown to increase the stability of SERCA2 mRNA and protein (32). Although a casual link remains to be established between the expression of antiapoptotic proteins and antioxidants, it is interesting to speculate that signaling may be initiated by the antioxidant properties of creatine (34).

Similarly, several investigations have suggested that expression of the cytosolic Ca^{2+}-buffering protein parvalbumin is under neural control. Denervation (22) reduces parvalbumin in rat fast-twitch muscles, whereas cross-reinnervation (42) of the fast-twitch extensor digitorum longus muscle and the slow-twitch soleus muscle results in decreases and increases, respectively, in parvalbumin. Application of a slow motoneuron-like, low-frequency impulse pattern (i.e., chronic low-frequency stimulation) to rabbit fast-twitch muscle induced a rapid decrease in parvalbumin content (31, 37). The decline in parvalbumin content in response to changes in contractile activity may be interpreted as a result of the fast-to-slow fiber type conversions (47), suggesting that parvalbumin content is under the control of fiber-type-specific programs. In contrast, our data clearly show that, in fast-twitch muscle, creatine loading alone severely reduced (Fig. 6) parvalbumin protein expression, which was not further reduced with running, as seen in the Con-Run group. Thus our findings indicate that parvalbumin protein expression is not purely dependent on neural regulation. In fact, it may only be indirectly influenced by neural regulation through changes in the intracellular energy potential that typically precede fast-to-slow fiber type transitions (18, 45). Because parvalbumin is largely restricted to type IId/x and

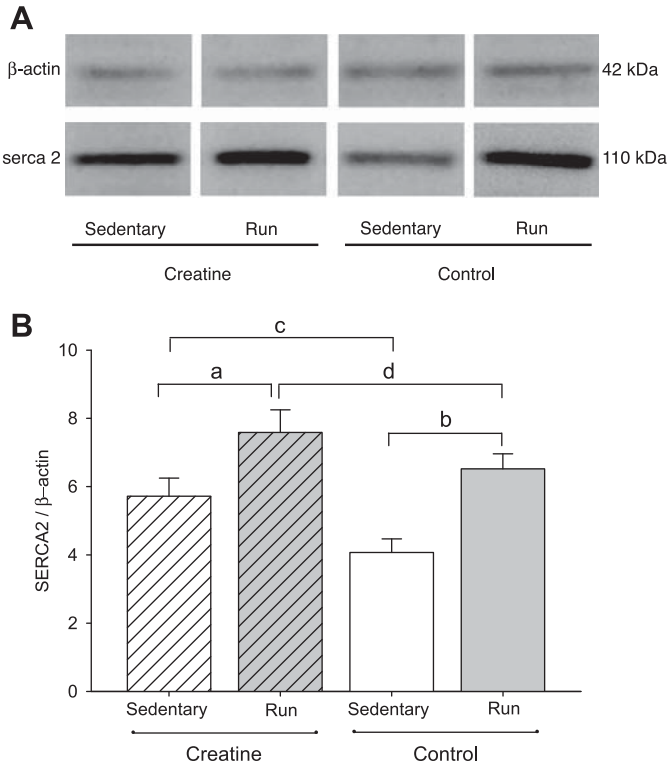

Fig. 7. *A*: representative immunoblot of SERCA2 in plantaris muscle. *B*: densitometric evaluation of *A* as ratio of SERCA2 to β-actin (loading control). See Fig. 3 legend for significance.

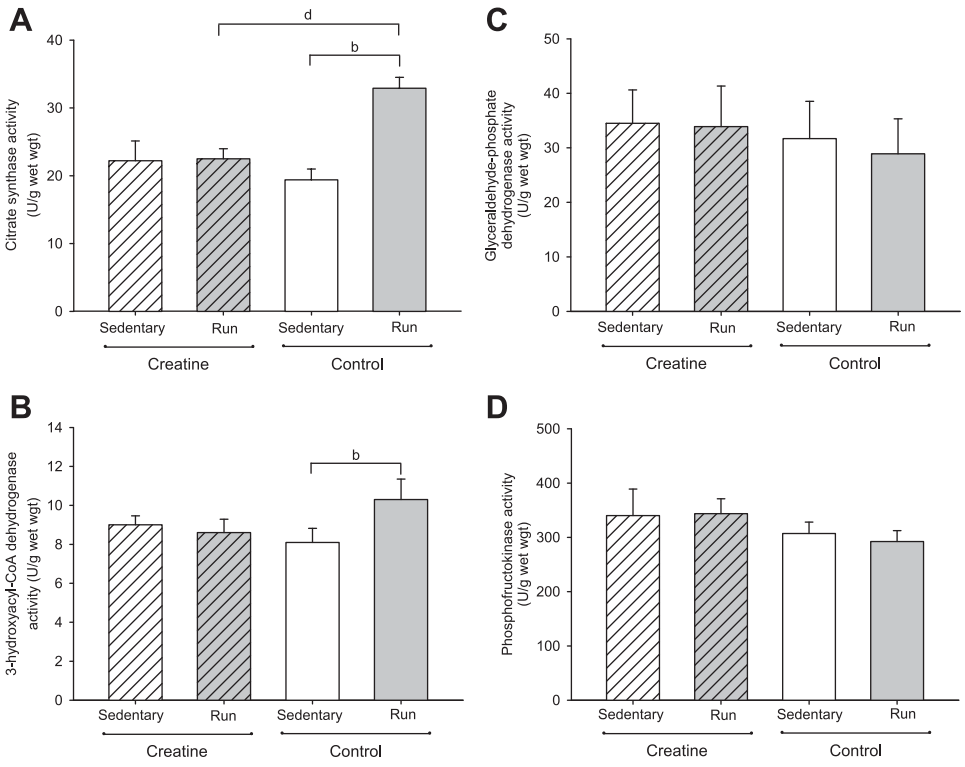

Fig. 8. Maximum activities of citrate synthase (EC 4.1.3.7; *A*), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35; *B*), GAPDH (EC 1.2.1.12; *C*), and phosphofructokinase (EC 2.7.1.11; *D*). See Fig. 3 legend for significance.

IIb fibers, the dramatic reduction in our study suggests that elevation of the capacity for high-energy shuttling alleviates the need for Ca^{2+} buffering by parvalbumin. Thus the intriguing possibility exists that the elevation of slow SERCA2 in response to creatine loading compensates by increasing the efficiency of Ca^{2+} reuptake. It also follows that enhanced Ca^{2+} reuptake would sufficiently maintain a lower free intracellular Ca^{2+} concentration (50) and, consequently, reduce the activation of calcineurin, a Ca^{2+}-dependent protein phosphatase, which stimulates slow-twitch fiber-specific gene promoters (11, 43).

Metabolic profile. The running-induced increases in maximal mitochondrial reference enzyme activities, CS and HADH, observed in the present study were similar in magnitude to those observed in previous reports of rodent run training (9, 20). However, reciprocal reductions in reference enzymes reflecting glycolytic capacity were not observed in the present study: levels remained unchanged after 13 wk of run training (9, 20). Our novel observation that creatine loading prevented a running-induced increase in mitochondrial content in the present study is intriguing. The increases in CS and HADH in response to run training alone are also similar to our recent findings that chronic activation of 5′-AMP-activated protein kinase (AMPK) by 5-aminoimidazole-4-carboxamide-1β-*d*-ribofuranoside induces similar changes, which reflect mitochondrial biogenesis, which is highly localized within the fast-twitch type IId/x and IIb fibers (2, 52). In contrast, the absence of an increase in CS or HADH within our creatine-loaded plantaris muscles after run training is consistent with inhibition of AMPK secondary to a greater average ATP-to-AMP ratio during running, as well as direct inhibition of AMPK due to elevated phosphocreatine (49). Indeed, it has been shown that energy deprivation within murine skeletal muscles by β-GPA feeding can induce substantial mitochondrial genesis in the presence of AMPK, but not in the muscles of transgenic mice that express an inactive dominant-negative mutant form of AMPK (68).

Perspectives and Significance

The results of the present study are consistent with the notion that creatine loading of rodent skeletal muscles not only improves fatigability, as seen in human studies, but also has the potential to promote increases in the proportion and CSA of the fastest-contracting type IIb fibers. These changes should result in a more powerful muscle fiber type that appears to be adequately supported by the existing glycolytic potential without changes in the capacity for terminal oxidation of carbohydrates and lipids. This probably relates to the greater rate of high-energy phosphate shuttling that is possible in the presence of increased intramuscular creatine, which would allow for more efficient movement of ATP produced by the glycolytic complex, located within each sarcomere, to neighboring contracting myofibrils. It is not likely that muscles containing high proportions of type I and IIa fibers and few type IId/x fibers, such as the human vastus lateralis (52), would benefit from creatine loading. In contrast, muscles with higher proportions of the fastest fiber type(s) (i.e., type IId/x or IIb fibers) typically seen in small mammals (45, 47) are most likely to benefit from creatine feeding during exercise training.

ACKNOWLEDGMENTS

The authors thank Z. Kenwell for valuable technical assistance.

GRANTS

This study was funded by research grants from the Natural Sciences and Engineering Council of Canada (NSERC) to C. T. Putman and the Alberta

Heritage Foundation for Medical Research (AHFMR) to C. T. Putman. M Gallo was supported by NSERC and Izaak Walton Killam Graduate Scholarships. K. J. B. Martins was supported by NSERC and AHFMR Graduate Scholarships. T. Gordon is an AHFMR Senior Investigator. C. T. Putman is a Heritage Senior Medical Scholar of AHFMR.

REFERENCES

1. **Allen DL, Harrison BC, Maass A, Bell ML, Byrnes WC, Leinwand LA.** Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. *J Appl Physiol* 90: 1900–1908, 2001.
2. **Bamford JA, Lopaschuk GD, MacLean IM, Reinhart ML, Dixon WT, Putman CT.** Effects of chronic AICAR administration on metabolic and contractile phenotype in rat skeletal muscle. *Can J Physiol Pharmacol* 81: 1072–1082, 2003.
3. **Bass A, Brdiczka D, Eyer P, Hofer S, Pette D.** Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Eur J Biochem* 10: 198–206, 1969.
4. **Bessman SP.** The creatine phosphate energy shuttle—the molecular asymmetry of a "pool." *Anal Biochem* 161: 519–523, 1987.
5. **Bigard X, Sanchez H, Zoll J, Mateo P, Rousseau V, Veksler V, Ventura-Clapier R.** Calcineurin coregulates contractile and metabolic components of slow muscle phenotype. *J Biol Chem* 275: 19653–19660, 2000.
6. **Bottinelli R, Betto R, Schiaffino S, Reggiani C.** Maximum shortening velocity and coexistence of myosin heavy chain isoforms in single skinned fast fibres of rat skeletal muscle. *J Muscle Res Cell Motil* 15: 413–419, 1994.
7. **Bottinelli R, Betto R, Schiaffino S, Reggiani C.** Unloaded shortening velocity and myosin heavy chain and alkali light chain isoform composition in rat skeletal muscle fibres. *J Physiol* 478: 341–349, 1994.
8. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
9. **Brannon TA, Adams GR, Conniff CL, Baldwin KM.** Effects of creatine loading and training on running performance and biochemical properties of rat skeletal muscle. *Med Sci Sports Exerc* 29: 489–495, 1997.
10. **Carroll S, Nicotera P, Pette D.** Calcium transients in single fibers of low-frequency stimulated fast-twitch muscle of rat. *Am J Physiol Cell Physiol* 277: C1122–C1129, 1999.
11. **Chin ER, Grange RW, Viau F, Simard AR, Humphries C, Shelton J, Bassel-Duby R, Williams RS, Michel RN.** Alterations in slow-twitch muscle phenotype in transgenic mice overexpressing the Ca^{2+} buffering protein parvalbumin. *J Physiol* 547: 649–663, 2003.
12. **Demirel HA, Powers SK, Naito H, Hughes M, Coombes JS.** Exercise-induced alterations in skeletal muscle myosin heavy chain phenotype: dose-response relationship. *J Appl Physiol* 86: 1002–1008, 1999.
13. **Dunn SE, Michel RN.** Differential sensitivity of myosin-heavy-chain-typed fibers to distinct aggregates of nerve-mediated activation. *Pflügers Arch* 437: 432–440, 1999.
14. **Eijnde BO, Lebacq J, Ramaekers M, Hespel P.** Effect of muscle creatine content manipulation on contractile properties in mouse muscles. *Muscle Nerve* 29: 428–435, 2004.
15. **Freyssenet D, Berthon P, Barthelemy JC, Busso T, Geyssant A, Denis C.** Energetic status and mitochondrial oxidative capacity of rat skeletal muscle in response to creatine analogue ingestion. *Biochim Biophys Acta* 1228: 211–215, 1995.
16. **Gallo M, Gordon T, Syrotuik D, Shu Y, Tyreman N, Maclean I, Kenwell Z, Putman CT.** Effects of long-term creatine feeding and running on isometric functional measures and myosin heavy chain content of rat skeletal muscles. *Pflügers Arch* 452: 744–755, 2006.
17. **Gallo M, Gordon T, Tyreman N, Shu Y, Putman CT.** Reliability of isolated isometric function measures in rat muscles composed of different fibre types. *Exp Physiol* 89: 583–592, 2004.
18. **Green HJ, Düsterhöft S, Dux L, Pette D.** Metabolite patterns related to exhaustion, recovery and transformation of chronically stimulated rabbit fast-twitch muscle. *Pflügers Arch* 420: 359–366, 1992.
19. **Green HJ, Klug GA, Reichmann H, Seedorf U, Wiehrer W, Pette D.** Exercise-induced fibre type transitions with regard to myosin, parvalbumin, and sarcoplasmic reticulum in muscles of the rat. *Pflügers Arch* 400: 432–438, 1984.
20. **Green HJ, Reichmann H, Pette D.** Fiber type specific transformations in the enzyme activity pattern of rat vastus lateralis muscle by prolonged endurance training. *Pflügers Arch* 399: 216–222, 1983.
21. **Greenhaff PL, Bodin K, Soderlund K, Hultman E.** Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *Am J Physiol Endocrinol Metab* 266: E725–E730, 1994.
22. **Gundersen K, Leberer E, Lomo T, Pette D, Staron RS.** Fibre types, calcium-sequestering proteins and metabolic enzymes in denervated and chronically stimulated muscles of the rat. *J Physiol* 398: 177–189, 1988.
23. **Hämäläinen N, Pette D.** Coordinated fast-to-slow transitions of myosin and SERCA isoforms in chronically stimulated muscles of euthyroid and hyperthyroid rabbits. *J Muscle Res Cell Motil* 18: 545–554, 1997.
24. **Hämäläinen N, Pette D.** Myosin and SERCA isoform expression in denervated slow-twitch muscle of euthyroid and hyperthyroid rabbits. *J Muscle Res Cell Motil* 22: 453–457, 2001.
25. **Harris RC, Soderlund K, Hultman E.** Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin Sci (Lond)* 83: 367–374, 1992.
26. **Huber B, Pette D.** Dynamics of parvalbumin expression in low-frequency-stimulated fast-twitch rat muscle. *Eur J Biochem* 236: 814–819, 1996.
27. **Hultman E, Soderlund K, Timmons JA, Cederblad G, Greenhaff PL.** Muscle creatine loading in men. *J Appl Physiol* 81: 232–237, 1996.
28. **Ingwall JS, Weiner CD, Morales MF, Davis E, Stockdale FE.** Specificity of creatine in the control of muscle protein synthesis. *J Cell Biol* 62: 145–151, 1974.
29. **Kandarian SC, Peters DG, Taylor JA, Williams JH.** Skeletal muscle overload upregulates the sarcoplasmic reticulum slow calcium pump gene. *Am J Physiol Cell Physiol* 266: C1190–C1197, 1994.
30. **Kariya F, Yamauchi H, Kobayashi K, Narusawa M, Nakahara Y.** Effects of prolonged voluntary wheel-running on muscle structure and function in rat skeletal muscle. *Eur J Appl Physiol* 92: 90–97, 2004.
31. **Klug G, Wiehrer W, Reichmann H, Leberer E, Pette D.** Relationships between early alterations in parvalbumins, sarcoplasmic reticulum and metabolic enzymes in chronically stimulated fast twitch muscle. *Pflügers Arch* 399: 280–284, 1983.
32. **Kuo TH, Kim HR, Zhu L, Yu Y, Lin HM, Tsang W.** Modulation of endoplasmic reticulum calcium pump by Bcl-2. *Oncogene* 17: 1903–1910, 1998.
33. **Lambert MI, Noakes TD.** Spontaneous running increases $\dot{V}O_{2max}$ and running performance in rats. *J Appl Physiol* 68: 400–403, 1990.
34. **Lawler JM, Barnes WS, Wu G, Song W, Demaree S.** Direct antioxidant properties of creatine *Biochem Biophys Res Commun* 290: 47–52, 2002.
35. **Leberer E, Hartner KT, Brandl CJ, Fujii J, Tada M, MacLennan DH, Pette D.** Slow/cardiac sarcoplasmic reticulum Ca^{2+}-ATPase and phospholamban mRNAs are expressed in chronically stimulated rabbit fast-twitch muscle. *Eur J Biochem* 185: 51–54, 1989.
36. **Leberer E, Hartner KT, Pette D.** Reversible inhibition of sarcoplasmic reticulum Ca-ATPase by altered neuromuscular activity in rabbit fast-twitch muscle. *Eur J Biochem* 162: 555–561, 1987.
37. **Leberer E, Pette D.** Neural regulation of parvalbumin expression in mammalian skeletal muscle. *Biochem J* 235: 67–73, 1986.
38. **Leeuw T, Pette D.** Coordinate changes in the expression of troponin subunit and myosin heavy-chain isoforms during fast-to-slow transition of low-frequency-stimulated rabbit muscle. *Eur J Biochem* 213: 1039–1046, 1993.
39. **Leeuw T, Pette D.** Coordinate changes of myosin light and heavy chain isoforms during forced fiber type transitions in rabbit muscle. *Dev Genet* 19: 163–168, 1996.
40. **Lowey S, Waller GS, Trybus KM.** Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature* 365: 454–456, 1993.
41. **Morse AD, Russell JC, Hunt TW, Wood GO, Epling WF, Pierce WD.** Diurnal variation of intensive running in food-deprived rats. *Can J Physiol Pharmacol* 73: 1519–1523, 1995.
42. **Muntener M, Rowlerson AM, Berchtold MW, Heizmann CW.** Changes in the concentration of the calcium-binding parvalbumin in cross-reinnervated rat muscles. Comparison of biochemical with physiological and histochemical parameters. *J Biol Chem* 262: 465–469, 1987.
43. **Naya FJ, Mercer B, Shelton J, Richardson JA, Williams RS, Olson EN.** Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J Biol Chem* 275: 4545–4548, 2000.
44. **Petrofsky JS, Fitch CD.** Contractile characteristics of skeletal muscles depleted of phosphocreatine. *Pflügers Arch* 384: 123–129, 1980.
45. **Pette D, Staron RS.** Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev Physiol Biochem Pharmacol* 116: 1–76, 1990.
46. **Pette D, Staron RS.** Mammalian skeletal muscle fiber type transitions. *Int Rev Cytol* 170: 143–223, 1997.

47. **Pette D, Staron RS.** Myosin isoforms, muscle fiber types, and transitions. *Microsc Res Tech* 50: 500–509, 2000.
48. **Pette D, Vrbová G.** Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation. *Rev Physiol Biochem Pharmacol* 120: 115–202, 1992.
49. **Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D.** Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J* 17: 1688–1699, 1998.
50. **Pulido SM, Passaquin AC, Leijendekker WJ, Challet C, Wallimann T, Ruegg UT.** Creatine supplementation improves intracellular Ca^{2+} handling and survival in mdx skeletal muscle cells. *FEBS Lett* 439: 357–362, 1998.
51. **Putman CT, Düsterhöft S, Pette D.** Satellite cell proliferation in low-frequency stimulated fast muscle of hypothyroid rat. *Am J Physiol Cell Physiol* 279: C682–C690, 2000.
52. **Putman CT, Kiricsi M, Pearcey J, MacLean IM, Bamford JA, Murdoch GK, Dixon WT, Pette D.** AMPK activation increases UCP-3 expression and mitochondrial enzyme activities in rat muscle without fibre type transitions. *J Physiol* 551: 169–178, 2003.
53. **Reichmann H, Srihari T, Pette D.** Ipsi- and contralateral fibre transformations by cross-reinnervation. A principle of symmetry. *Pflügers Arch* 397: 202–208, 1983.
54. **Ren JM, Ohira Y, Holloszy JO, Hämäläinen N, Traub I, Pette D.** Effects of β-guanidinopropionic acid-feeding on the patterns of myosin isoforms in rat fast-twitch muscle. *Pflügers Arch* 430: 389–393, 1995.
55. **Rodnick KJ, Reaven GM, Haskell WL, Sims CR, Mondon CE.** Variations in running activity and enzymatic adaptations in voluntary running rats. *J Appl Physiol* 66: 1250–1257, 1989.
56. **Roussel D, Lhenry F, Ecochard L, Sempore B, Rouanet JL, Favier R.** Differential effects of endurance training and creatine depletion on regional mitochondrial adaptations in rat skeletal muscle. *Biochem J* 350: 547–553, 2000.
57. **Roy BD, Bourgeois JM, Mahoney DJ, Tarnopolsky MA.** Dietary supplementation with creatine monohydrate prevents corticosteroid-induced attenuation of growth in young rats. *Can J Physiol Pharmacol* 80: 1008–1014, 2002.
58. **Schiaffino S, Gorza L, Pitton G, Saggin L, Ausoni S, Sartore S, Lomo T.** Embryonic and neonatal myosin heavy chain in denervated and paralyzed rat skeletal muscle. *Dev Biol* 127: 1–11, 1988.
59. **Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lomo T.** Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J Muscle Res Cell Motil* 10: 197–205, 1989.
60. **Schwaller B, Dick J, Dhoot G, Carroll S, Vrbová G, Nicotera P, Pette D, Wyss A, Bluethmann H, Hunziker W, Celio MR.** Prolonged contraction-relaxation cycle of fast-twitch muscles in parvalbumin knockout mice. *Am J Physiol Cell Physiol* 276: C395–C403, 1999.
61. **Srere PA.** Citrate synthase. In: *Citric Acid Cycle*, edited by Lowenstein JM. New York: Academic, 1969, p. 3–11.
62. **Sullivan VK, Powers SK, Criswell DS, Tumer N, Larochelle JS, Lowenthal D.** Myosin heavy chain composition in young and old rat skeletal muscle: effects of endurance exercise. *J Appl Physiol* 78: 2115–2120, 1995.
63. **Towbin H, Staehelin T, Gordon J.** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76: 4350–4354, 1979.
64. **Van Deursen J, Jap P, Heerschap A, ter Laak H, Ruitenbeek W, Wieringa B.** Effects of the creatine analogue β-guanidinopropionic acid on skeletal muscles of mice deficient in muscle creatine kinase. *Biochim Biophys Acta* 1185: 327–335, 1994.
65. **Wallimann T, Dolder M, Schlattner U, Eder M, Hornemann T, O'Gorman E, Ruck A, Brdiczka D.** Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *Biofactors* 8: 229–234, 1998.
66. **Zador E, Fenyvesi R, Wuytack F.** Expression of SERCA2a is not regulated by calcineurin or upon mechanical unloading in skeletal muscle regeneration. *FEBS Lett* 579: 749–752, 2005.
67. **Zador E, Wuytack F.** Expression of SERCA2a is independent of innervation in regenerating soleus muscle. *Am J Physiol Cell Physiol* 285: C853–C861, 2003.
68. **Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI.** AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci USA* 99: 15983–15987, 2002.