Nerve Activity-dependent Modulation of Calcineurin Signaling in Adult Fast and Slow Skeletal Muscle Fibers*

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Skeletal muscle fibers display considerable variation in their size and expression of contractile proteins. Slow/type I fibers display relatively small diameters and express slow isoforms of myofibrillar and Ca²⁺-regulatory proteins, whereas fast/type II fibers generally display larger fiber girths and express faster isoforms of these proteins (see Ref. 1 for review). The phenotype of an adult muscle fiber is malleable and is largely influenced by the amount and pattern of activity that it receives from its motor nerve (1). Compelling evidence of this is provided by studies that have induced transformations in muscle phenotype by cross-reinnervating slow or fast muscles with their foreign nerve counterparts or by electrically stimulating these muscles with impulse patterns modeled after foreign nerve activity and positively correlated with muscle usage under normal weightbearing conditions. With increased nerve-mediated activity, calcineurin dephosphorylation of these targets was found to be potentiated in a way that paralleled the higher muscle activation profiles associated with functional overload or nerve electrical stimulation conditions. We also establish that muscle activity must be sustained above native levels for calcineurin-dependent dephosphorylation of MEF2A and MEF2D to be transduced into an increase in MEF2 transcriptional function, suggesting that calcineurin cooperates with other activity-linked events to signal via these proteins. Finally, examination of individual fiber responses to overload and nerve electrical stimulation revealed that calcineurin-MEF2 signaling occurs in all fiber types but most readily in fibers that are normally least active (i.e. those expressing IIx and IIb myosin heavy chain (MHC)), suggesting that signaling via this phosphatase is also dependent upon the activation history of the muscle cell.

This study tested the hypothesis that calcineurin signaling is modulated in skeletal muscle cells by fluctuations in nerve-mediated activity. We show that dephosphorylation of NFATc1, MEF2A, and MEF2D transcription factors by calcineurin in all muscle types is dependent on nerve activity and positively correlated with muscle usage under normal weightbearing conditions. With increased nerve-mediated activity, calcineurin dephosphorylation of these targets was found to be potentiated in a way that paralleled the higher muscle activation profiles associated with functional overload or nerve electrical stimulation conditions. We also establish that muscle activity must be sustained above native levels for calcineurin-dependent dephosphorylation of MEF2A and MEF2D to be transduced into an increase in MEF2 transcriptional function, suggesting that calcineurin cooperates with other activity-linked events to signal via these proteins. Finally, examination of individual fiber responses to overload and nerve electrical stimulation revealed that calcineurin-MEF2 signaling occurs in all fiber types but most readily in fibers that are normally least active (i.e. those expressing IIx and IIb myosin heavy chain (MHC)), suggesting that signaling via this phosphatase is also dependent upon the activation history of the muscle cell.

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Acetylcholine is released from the nerve terminal with each motoneuron discharge. The binding of this neurotransmitter to receptor-mediated ion channels on the target muscle cell induces a depolarization of the sarcolemma and triggers the release of Ca²⁺ from the sarcoplasmic reticulum. It is suggested that as a result of their distinct discharge profiles, slow and fast motoneurons modulate the expression of muscle genes via the different patterns of intracellular Ca²⁺ that they evoke (7). Frequent muscle depolarizations elicited in response to the tonic activity of slow motoneurons induce a sustained elevation of muscle Ca²⁺ (8), whereas the infrequent, burst firing of fast motoneurons evokes transient spikes in muscle Ca²⁺ (9).

Calcineurin, a Ca²⁺/calmodulin (CaM)-activated phosphatase, has been implicated as a molecular decoder of sustained Ca²⁺ signals evoked in muscle cells in response to frequent nerve-mediated depolarizations and as an integral signaling intermediate in pathways that promote skeletal muscle fiber hypertrophy and expression of a slower contractile protein phenotype (for review, see Ref. 10). It is postulated that nerve-mediated increases in muscle intracellular Ca²⁺ activate calcineurin and CaM-sensitive kinases which in turn trigger the action of nuclear factor of activated T cells c1 (NFATc1) and myocyte enhancer factor 2 (MEF2) proteins (7, 10, 11). It is thought that these proteins bind cooperatively with other transcription factors such as GATA-2 to activate the transcription of slow fiber-specific or growth-regulatory genes (7, 10, 11). In support of this model, increasing intracellular Ca²⁺ levels in cultured myocytes with ionomycin activates calcineurin (12, 13) and promotes the acquisition of a slow oxidative phenotype (13, 14). Moreover, NFAT and MEF2 binding appears essential for the calcineurin-dependent activation and slow fiber-specific expression of the slow upstream regulatory element enhancer, a transcriptional element that directs the expression of the

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1 The abbreviations used are: CaM, calmodulin; OV, overload; NFAT, nuclear factor of activated T cells c1; MEF2, myocyte enhancer factor 2; TTX, tetrodotoxin; CaMK, Ca²⁺/calmodulin-dependent kinase; EMG, electromyography; CsA, cyclosporin A; AP, alkaline phosphatase; IGF, insulin-like growth factor; S, slow; PR, fast fatigue-resistant; FI, fast fatigue-intermediate; FF, fast fatigable; PBS, phosphate-buffered saline; MHC, myosin heavy chain; β-gal, β-galactosidase; GFP, green fluorescent protein.
troponin I slow gene (11). The clear importance of calcineurin signaling events in regulating muscle phenotype is further demonstrated by experiments that prevented fiber growth and fast-to-slow fiber type transitions (i.e. IIb → IIX → IIa → I) in the overloaded (OV) plantaris (15), and induced a subtle atrophy and shift toward expression of faster contractile proteins in the normal weightbearing soleus (7, 16), by administration of the calcineurin inhibitors cyclosporin A (CsA) or FK506. Although the importance of Ca\(^{2+}\) and calcineurin-dependent signaling pathways in regulating fiber phenotype is established, the role of nerve-mediated activity as an in vivo upstream modulator of calcineurin activity in skeletal muscles has yet to be confirmed.

In the present study, we thus tested the hypothesis that calcineurin signaling pathways in skeletal muscle cells are sensitive to nerve-mediated activity. To this end, we investigated whether calcineurin is more extensively activated in more highly recruited muscles under normal weightbearing conditions and whether this activity is countered by neuronal quiescence. Next, we examined the potentiation of calcineurin signaling in overloaded plantaris muscles over the time course when motor unit recruitment levels are rapidly doubled by this condition (17). Moreover, to provide insight into what specific aspect of the nerve electrical stimulus (i.e. pulse frequency, amount, etc.) is key to activating calcineurin in muscle cells, we contrasted the effectiveness of various exogenous nerve stimulation paradigms to initiate signaling via this enzyme. The extent of muscle calcineurin signaling was assessed by measuring the phosphorylation status of the calcineurin substrates NFATc1, MEF2A, and MEF2D; the nuclear abundance of NFATc1; and the transcriptional function of MEF2.

Consistent with our hypothesis, we show calcineurin-mediated dephosphorylation of NFATc1, MEF2A, and MEF2D to be nerve activity-dependent and to occur in all muscle types. Dephosphorylation of these signaling factors by calcineurin appeared to be positively correlated with muscle usage under normal weightbearing conditions. Moreover, we provide evidence that muscle activity must be increased above native levels for calcineurin-dependent dephosphorylation of MEF2A or MEF2D to be transduced into an increase in transcriptional function of these proteins, suggesting that accessory activity-linked signaling events are required for complete signaling of calcineurin to target genes (18). Finally, we found that calcineurin-MEF2D signaling was initiated in all muscle fiber types in response to increased nerve-mediated activity, but occurred most readily in the least active fibers, those expressing IIx or Iib MHC, suggesting that modulation of this pathway is also influenced by the activation history of each muscle cell.

**EXPERIMENTAL PROCEDURES**

**Animals, Surgeries, and Stimulation Paradigms—**Sprague-Dawley rats (260–300 g) and CD-1 mice (25–35 g) were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Tg mice (C57BL6) harboring lacZ under the control of three copies of a high affinity desmin MEF2b consensus element (desMEF2) have been described previously (19). All surgical procedures were performed under aseptic conditions on animals anesthetized by intramuscular injection (1.2 μg/kg) of 100 mg/ml ketamine hydrochloride and 10 mg/ml xylazine in a volume ratio of 1:6.1. Treatment of animals was in accordance with the guidelines established by the Canadian Council on Animal Care. For denervation and TTX paralysis experiments, hamstring musculature in the left hip region of rats was incised to expose the sciatic nerve. In denervated animals, a 5-mm portion of the sciatic nerve was excised. In TTX animals, muscles in the left hindlimb were paralyzed for 3 or 7 days by chronic superfusion of the sciatic nerve (0.5 μl/h) with this sodium channel blocker (350 μg/ml) via a mini-osmotic pump (model 1007D, Alza Corp., Palo Alto, CA) and attached silicon drug delivery system as described in detail previously (5). Briefly, a silicon cuff was secured around the nerve and was connected by silicon tubing to the pump that was implanted subcutaneously on the animal’s back. The efficacy of paralysis of the triceps surae muscles in TTX animals was verified twice daily using established ankle reflex criteria (5). Control rats were implanted with a sham nerve cuff and drug delivery system. For OV experiments, compensatory hypertrophy of the plantaris was induced in each hindlimb of desMEF2 Tg and CD-1 mice by surgically ablating the soleus and a major portion of the gastrocnemius muscle (15). In control mice, the tendons of the soleus and gastrocnemius were separated from the plantaris tendon but were not severed. For muscle regeneration experiments, local injury of the plantaris in each hindlimb of CD-1 mice was induced via freezing the muscle. Briefly, the medial hamstrings musculature was incised to expose the distal half of the plantaris, and equal lengths of the muscle and overlying fascia were then applied to the surface of the muscle for 10 s. For electrical stimulation experiments, hamstring musculature in the left hip region of each animal was incised to expose the sciatic nerve. Hindlimb muscles were activated by electrically stimulating the sciatic nerve at supramaximal voltage (15 V) with a bipolar electrode using a 10-Hz continuous or an intermittent 100-Hz stimulus pattern (Grass® Stimulator, model S48, Grass Instrument, Quincy, MA). For the latter pattern, 1–100-Hz trains were administered once every 30 s, 1 min, 2 min, 5 min, 15 min, or 30 min. Administered stimuli were 0.1-mS square wave pulses. Hip, knee, and ankle joints were fixed during the stimulation session to ensure that contractions were isometric.

**CsA and FK506 Administration and Muscle Removal—**For OV and electrical stimulation experiments, mice were intraperitoneally injected with either CsA (25 mg/kg), FK506 (3 mg/kg), or vehicle (Cremophor® EL; Sigma-Aldrich, Oakville, Ontario, Canada) twice daily, separated by a 12-h interval, for 1–28 days. Injections were initiated 1 day prior to the beginning of each experiment. Vials of CsA (50 mg/ml; Novartis, Dorval, Quebec, Canada) were mixed in a ratio of 1:7 (v/v) with vehicle, whereas FK506 was solubilized in ethanol (5 μg/ml drug) and then diluted to a final concentration of 0.75 mg/ml with vehicle. To investigate the long term effect of calcineurin inhibition on the phosphorylation status of NFATc1 and MEF2 in normal weightbearing hindlimb muscles, rats were injected with CsA (10 mg/kg, n = 3) or vehicle (n = 3) twice daily, or FK506 (2 mg/kg, n = 2) once daily, for a period of 28 days. For the latter experiment, blood was collected for analyses of CsA and FK506 levels (London Health Science Center, London, Ontario, Canada). After each experimental condition, muscles were excised and quick-frozen in melting isopentane pre-cooled with liquid nitrogen. The 25 mg/kg dose of CsA in mice produced blood levels of this drug that were comparable with those obtained using a 10 mg/kg dose in rats (2865 ± 517 nmol/ml in mice versus 2157 ± 613 nmol/ml in rats) and resulted in a ~60% decrease in total plantaris calcineurin activity (17.4 ± 0.6 pmol/mg protein in vehicle-treated versus 7.1 ± 0.9 pmol/mg protein in CsA-treated mice, n = 3) as measured by IsoTechnika (Edmonton, Alberta, Canada). Administration of FK506 resulted in lower blood levels in rats (13.0 ± 1.7 ng/ml) than in mice (83 ± 14 ng/ml). This lower dosage of FK506 in rats was necessary to ensure the maintenance of the health and body weight of these animals for the duration of the treatment period.

**Western Blot Analysis of MEF2 and NFATc1 Expression—**Whole cell and nuclear extracts were generated as described previously (18). For in vitro alkaline phosphatase (AP) reactions, muscle samples (150 mg) were homogenized in 1 ml of HEPES buffer (4 mM EGTA, 10 mM EDTA, 0.1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride in 50 mM HEPES, pH = 7.4), incubated on ice for 1 h, and then centrifuged for 15 min at 20,000 × g. Each lysate (50 μg) was incubated with 0, 100, or 200 units of AP covalently linked to agarose beads (Sigma-Aldrich) in AP reaction buffer (1 mM MgCl\(_2\), 50 mM Tris-HCl, pH = 8.5) at 30 °C for 15 min. The beads were separated from the reaction mixture by centrifugation, the supernatant recovered, and then concentrated to the original volume of added protein using a 5000 nominal molecular weight limit 4-ml Ultrafres® filter unit (Millipore, Bedford, MA). The concentration of total protein in whole cell nuclear extract, and AP-treated samples was estimated with the Bradford Microassay (Bio-Rad, Mississauga, Ontario, Canada) using bovine serum albumin as a standard.

Samples were each diluted in two volumes of 2× Laemmlı Buffer (Bio-Rad), boiled for 3–5 min, and run on a 6–8% SDS-polyacrylamide gel using the MiniProtean III system (Bio-Rad). Proteins were stained with Coomassie Blue to visualize loading of samples. Gels were transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) and stained with Ponceau S Red to verify equal transfer efficiency. Immunoblotting was performed using standard procedures with working dilutions of the following primary antibodies: NFATc1 (Affinity Bioreagents, Inc., Golden, CO), MEF2A (Santa Cruz Biotechnology Inc., Santa Cruz, CA),
and MEF2D (gift from Dr. R. Prywes). The MEF2D antibody cross-reacts with MEF2C and was used to estimate the abundance of this protein in whole cell and nuclear extracts. Membranes were subsequently probed with horseradish peroxidase secondary antibody conjugates (Sigma), and labeled proteins were visualized using the ECL plus system (Amersham, Arlington Heights, IL). The molecular weight of each protein was estimated using a Kilopeptide broad range protein standard (Bio-Rad). MEF2C was used as a gel loading control for normal weightbearing, denervated, TTXX-inactivated, and electrical stimulation samples, because the expression of this protein did not differ between muscle types or across these different treatment conditions. For UV experiments, Coomassie blue-stained SDS-polyacrylamide gels were used as loading controls, because MEF2C protein content was found to increase over the time course of this condition (data not shown).

Quantification of NFATc1, MEF2A, and MEF2D Phosphorylation—We used AP-treated samples to determine which bands were best suited to measure the phosphorylation status of NFATc1, MEF2A, and MEF2D proteins. Up to 6 NFATc1 bands were detected ranging in molecular mass from 85 to 141 kDa (Fig. 1A, see AP panels) in untreated samples. Pre-treatment of protein extracts with AP increased the prevalence of the 85- and 93-kDa species of NFATc1 (Fig. 1A, closed arrows), suggesting that detected bands correspond to dephosphorylated (85 and 89 kDa) and phosphorylated (>89 kDa) forms of this protein. Stimulation with treatment reduced both MEF2A and MEF2C bands to single 70- and 67-kDa species, respectively (Fig. 1A, open arrows, closed arrows). The density of phosphorylated (dePO4) and most phosphorylated (most PO4, band(s) of NFATc1, MEF2D, or MEF2A was determined using AlphaEaseFC™/FluoroChem software (Alpha Innotech Corp., San Leandro, CA) and expressed relative to the summed density of all bands for that sample.

RESULTS

Calcineurin Dephosphorylation of NFATc1 and MEF2 Is Correlated with Muscle Usage in Normal Weightbearing Muscles—To test the hypothesis that calcineurin signaling is modulated by nerve-mediated activity, we first investigated whether the calcineurin substrates NFATc1, MEF2A, or MEF2D are dephosphorylated to a greater extent in the soleus, a more highly recruited (20) and relatively slower (i.e. equal proportions of fibers expressing I or IIa MHC) muscle, versus its synergist, the medial gastrocnemius, a comparatively less active (20), faster (i.e. proportionately larger number of fibers expressing IIx or IIb MHC) plantar flexor under normal weightbearing conditions. Western blots of whole cell extracts showed a multiple banding pattern for each of these transcriptions. We identified dephosphorylated (dePO4, closed arrows) and most phosphorylated (most PO4, open arrows) forms of these proteins (Fig. 1A). All of these calcineurin substrates were more extensively dephosphorylated (Fig. 1, A and B) and expressed at higher levels relative to total protein (Fig. 1A) in the soleus compared with the medial gastrocnemius under normal weightbearing conditions. Compared with the soleus, the medial gastrocnemius displayed at least 2-fold higher levels of the most PO4 form of all three substrates and approximately half the levels of the dePO4 form of NFATc1 and MEF2D (Fig. 1B). For MEF2A, the divergence in banding patterns between these muscles was limited to the higher molecular weight species of this protein. The soleus also displayed higher amounts of NFATc1 in nuclear extracts compared with its less active synergist (Fig. 1C). Interestingly, NFATc1 detected in the nuclei from both muscles displayed multiple bands, consistent with the notion that partial dephosphorylation of this protein is sufficient to induce its nuclear import (18).

To investigate whether the enhanced dephosphorylation and expression of MEF2A and MEF2D observed in more active muscles (i.e. the soleus) was associated with an increase in transactivational function of MEF2, we used Tg mice (des-MEF2) that express a lacZ reporter gene driven by three high affinity MEF2 cis-elements from the desmin promoter (11, 19). These DNA binding sites do not preferentially bind one specific MEF2 isoform (19) and thus report the combined transactivational function of all MEF2 proteins. In contrast to a previous report of positive MEF2 transcriptional activity in ~15% of the solei from mice of this line (11), and detection of β-gal in Western blots of pooled (n = 5–7 muscles/lane) soleus samples, we did not observe any positive staining for β-gal activity under normal weightbearing conditions in any soleus cross-sections of the 15 des-MEF2 Tg mice examined, despite the fact that all of these mice displayed potential for expression of the transgene (data not shown; see “Experimental Procedures”). Expression

2 H. Wu, R. Bassel-Duby, and R. S. Williams, unpublished observations.
of the MEF2 reporter was not detected in cross-sections of normal weightbearing plantaris and medial gastrocnemius muscles, nor in Western blots of plantaris, EDL, and white vastus muscles of these Tg mice. These findings thus suggest that dephosphorylation of MEF2A and MEF2D by calcineurin does not necessarily lead to an increase in MEF2 transcriptional function in normal weightbearing muscles.

To address whether the greater dephosphorylation of NFATc1, MEF2D, and MEF2A in more active versus less active muscles was calcineurin-dependent, we investigated the influence of calcineurin inhibitors on the phosphorylation status of these transcription factors in the rat soleus and medial gastrocnemius. Consistent with a greater activation of calcineurin in the soleus, inhibition of this phosphatase by treatment of rats with either CsA or FK506 enhanced the phosphorylation of NFATc1 in this muscle, but not in the medial gastrocnemius (Fig. 2A). This was characterized in the soleus by an accumulation (p < 0.05) of the most PO4 form of NFATc1 (9 ± 3%, 31 ± 0.5%, and 27 ± 8% in vehicle, CsA, and FK506, respectively) and a reduction (p < 0.05) of the dePO4 form (22 ± 1%, 4 ± 0.3%, and 2 ± 1% in vehicle, CsA, and FK506, respectively), approaching the range of values seen in the normal weightbearing medial gastrocnemius (Fig. 2A). Of note, CsA treatment also induced a subtle phosphorylation of NFATc1 in the mouse plantaris (Fig. 5A), a fast twitch muscle that performs less mechanical work than the soleus, but more work than the gastrocnemius during locomotion (21). On the other hand, administration of calcineurin inhibitors enhanced the phosphorylation of MEF2D and MEF2A in both the soleus and medial gastrocnemius, suggesting that these calcineurin substrates may be preferentially sensitive to the activation of this phosphatase. For MEF2D, this was characterized by a 6-fold (p < 0.05) increase in the most PO4 form of this protein in the soleus and a 40% increase in the medial gastrocnemius (Fig. 2A, open arrows). Although, unlike the mouse, a major difference in MEF2A phosphorylation between normal weightbearing rat soleus and medial gastrocnemius muscles was not observed, CsA or FK506 treatment induced the appearance of the most PO4 form of this protein in both muscle types (Fig. 2A). Taken together, these results suggest that calcineurin activity is positively correlated with muscle usage and emphasize that signaling via this enzyme is not restricted to muscles displaying a slow phenotype under normal weightbearing conditions.

Nerve-dependent Regulation of Calcineurin—To verify that activation of calcineurin in normal weightbearing muscles was nerve activity-dependent, we investigated whether neuronal quiescence would mimic the effects of CsA and FK506, and
enhance the phosphorylation of calcineurin substrates. To this end, the sciatic nerve was either severed or chronically superfused with the sodium channel blocker TTX. Pharmacological blockade of nerve action potentials with TTX for either 3 (Fig. 2) or 7 days (data not shown) mirrored the effect of calcineurin inhibition and induced a subtle increase in the most PO4 form of NFATc1 in the soleus (five of six experiments) and a more prominent phosphorylation of MEF2D in both muscle types (4 of 6 solei and 3 of 5 medial gastrocnemius). These results thus confirm that calcineurin-mediated dephosphorylation of these substrates in normal weightbearing muscles is nerve activity-dependent. In contrast, denervation for 3 (Fig. 2) or 7 days (data not shown) did not influence the phosphorylation status of these transcription factors in the soleus or medial gastrocnemius. The failure of denervation to produce the same response as TTX-inactivation may be related to the fact that, unlike TTX, denervation induces marked muscle fibrillations (22) and is associated with increased leakiness of the sarcoplasmic reticulum and higher intracellular Ca2+ (23), which would presumably activate calcineurin independent of nerve activity.

**Calcineurin-MEF2 Signaling during Overload**—During functional compensatory OV, the plantaris sustains a rapid doubling of its activity levels as measured by EMG (17). This increase occurs gradually during the first 15 days of OV and then stabilizes at levels that are 2-fold greater than control over 30 days of this condition (17). To address whether the OV-induced enhancement of the calcineurin dephosphorylation of MEF2A or MEF2D (18) paralleled the time course of this increase in muscle recruitment, we assessed the phosphorylation status of these proteins over 21 days of this condition. Plantaris OV induced a progressive dephosphorylation of both MEF2A and MEF2D compared with sham-operated controls, evidenced by a gradual decrease in the relative amount of the most PO4 form that closely matched the time course of increased plantaris EMG with this condition (17) (Fig. 3B). OV also induced a progressive increase in MEF2D protein content, whereas the expression of MEF2A was transiently higher at 5–7 days of OV, returning near control levels by 14 days of this condition (Fig. 3A). These adaptations in MEF2A and MEF2D in response to OV appeared mediated by calcineurin, as they were either fully (i.e. MEF2D) or largely (i.e. MEF2A) prevented in mice treated with CsA or FK506 (Fig. 3A, right panels). The matching of dephosphorylation of MEF2A and MEF2D with OV muscle activation...
profiles (17) suggests that signaling of calcineurin to each of these transcription factors is mediated by the increase in muscle fiber recruitment associated with this condition. On the other hand, the difference in timing of MEF2A and MEF2D responses to OV suggests that signaling of calcineurin to each of these proteins may be subject to distinct regulatory mechanisms (i.e. activity versus increases in insulin-like growth factor, etc.).

The absence of MEF2 reporter gene expression in cross-sections of normal weightbearing muscles, despite a clear calcineurin-dependent dephosphorylation of MEF2A and MEF2D, raised the possibility that complete signaling of this phosphatase via these proteins may only occur when muscle activation is above normal levels. To test this notion, we investigated whether OV would induce an increase in MEF2 transcriptional function in the plantaris. Indeed, OV rapidly (within 2 days) induced the expression of the MEF2 reporter in a large number of plantaris fibers (Fig. 3, C–E). Peak expression of the MEF2 reporter occurred in plantaris cells at 5–7 days of OV and was largely compromised in mice treated with calcineurin inhibitors (Fig. 3E), thus mirroring the adaptations in MEF2A protein expression at this time (Fig. 3A). Positive β-gal staining was detected in a modest number of fibers at 14 or 21 days of OV (Fig. 3E), thus matching the persistent dephosphorylation of MEF2A and MEF2D in the longer term of this condition (Fig. 3, A and B).

In accordance with principles related to motor unit recruitment (24), a majority of the increase in activity in overloaded muscles is sustained by smaller, lower threshold slow (S) and fast fatigues-resistant (FR) motor units (respectively associated with fibers expressing I and IIa MHC). OV also results in a doubling of the normal recruitment levels of larger, usually silent, higher threshold fast fatigues-intermediate (FI) and fast fatigable (FF) motor units (respectively associated with fibers expressing IIX and IIB MHC). To assess whether MEF2 transcriptional function was increased in response to OV and followed the general recruitment order (S, type I) → (FR, type IIa) → (FI, IIX) → (FF, IIB), we examined the MHC phenotype of β-gal-positive cells in OV muscle cross-sections of desMEF2 Tg mice. At the onset of this condition (2 days), the MEF2 reporter was detected in all three subsets of fast fibers, particularly in those expressing IIX MHC (Fig. 4A). This finding suggests that the largest, least active fiber types (i.e. those expressing IIX or IIB) are initially the most responsive to increases in muscle activation. From day 5 to day 21 of OV, the majority of cells that were positive for β-gal activity were the small, highly recruited IIa fibers (Fig. 4A; compare B and C), although a significant number of positive IIX and IIB fibers were still detected. Taken together, these data suggest that the most important determinant of significant and sustained MEF2 transactivating function with OV appears to be the nerve-mediated imposition of fiber recruitment levels substantially above native levels relative to each cell type. Finally, the finding that expression of the MEF2 reporter was only detected in rare (~1%) nascent fibers (n = 84 ± 33/muscle) that are characteristic of 5-day OV muscles (compare Fig. 4, F and G) emphasizes that the activity-dependent induction of calcineurin-MEF2 signaling is a phenomenon restricted to mature muscle fibers.

In the latter series of experiments, we were unable to assess whether an increase in activity above normal would induce calcineurin-MEF2 signaling in type I fibers, because these cells are extremely rare in the plantaris in this line of Tg mice. Indeed, only 1 of 33 desMEF2 Tg mice examined displayed type I fibers in the plantaris, and none stained positive for β-gal activity. Thus, in a separate set of experiments, we investi-
to the significant OV-related dephosphorylation of NFATc1 at 2–5 days. To this end, we induced fiber regeneration in the distal half of the plantaris via freeze injury and subsequently examined the phosphorylation and nuclear localization of NFATc1 in the injured portion of this muscle at 3 days after injury. We found that regeneration induced a significant dephosphorylation of NFATc1 that matched the response observed after 2–5 days of OV (Fig. 5, compare A and J). Regenerating muscle fibers also displayed a prominent nuclear localization of NFATc1-GFP (Fig. 5, compare H and I). These data suggest the enhanced dephosphorylation of NFATc1 that occurred in the short-term of OV may be related in part to the OV-induced appearance of these nascent fibers. Of note, NFATc1 was not the only target of calcineurin in regenerating fibers, because an enhanced dephosphorylation of MEF2A and MEF2D was also observed in extracts from freeze-injured muscles (Fig. 5J). On the other hand, involvement of MEF2 in the regeneration process requires further examination, because expression of the MEF2 reporter was not detected in regenerating portions of the remnant gastrocnemius in desMEF2 Tg mice after 5 days of plantaris OV (data not shown). Taken together, these findings suggest that NFATc1 may have a dual role in OV muscles: one related to nerve-mediated signaling and the other to the establishment of nascent fiber phenotype.

Activation of Calcineurin by Nerve Electrical Stimulation

To provide insight into what specific aspect of the nerve electrical stimulus (i.e. pulse frequency, aggregate amount, etc.) is key to activating this phosphatase in muscle cells, we compared the ability of various nerve electrical stimulation paradigms to dephosphorylate NFATc1 and MEF2 proteins in the soleus and medial gastrocnemius. In contrast to OV, which imposes the largest increases of activity on smaller, low thresh-
old motor units, electrical stimulation of the sciatic nerve at supramaximal voltage simultaneously activates all motor unit types. We found that activation of the mouse sciatic nerve for 1 h with 10-Hz continuous stimuli, a frequency typical of S motor units, resulted in a calcineurin-dependent dephosphorylation of MEF2D in the fast medial gastrocnemius (Fig. 6A, top). Interestingly, a similar MEF2D response occurred when this muscle was activated intermittently for 1 h with bursts of high frequency (100 Hz) stimuli, a pulse frequency native to FF motor units (Fig. 6A, top), but only when these bursts were administered in close enough succession (interburst interval ≤ 1 min) (Fig. 6B). Consistent with MEF2D dephosphorylation findings, we found that electrical stimulation of desMEF2 Tg mice for 30 min, three times daily (each session separated by 3 h) with either the 10-Hz continuous or 100-Hz/30 s paradigms consistently induced an increase in MEF2 transcriptional activity in some fibers expressing IIb MHC in the medial gastrocnemius (Fig. 6, compare C and D). These paradigms also induced expression of the MEF2 reporter in some fibers expressing IIb in the plantaris (Fig. 6E). Given that the aggregate activity evoked by these paradigms (1 h and 200 s, respectively) largely exceeds the mean activity per hour of FF motor units (1.4–8.0 s/h; Ref 2) and associated IIb fibers, these data thus suggest that calcineurin-MEF2D signaling in fibers expressing this MHC is initiated when the aggregate amount of activity exceeds normal levels or when the interval between activation bursts is sufficiently brief (≤ 1 min). In contrast to MEF2D findings, neither paradigm of stimulation was effective in dephosphorylating MEF2A or NFATc1 in the medial gastrocnemius muscle (Fig. 6A, middle panels), even after several hours (data not shown), suggesting that MEF2D is relatively more sensitive to nerve-mediated dephosphorylation via calcineurin.

In contrast to findings in the medial gastrocnemius, nerve-mediated activation of the soleus for 1–2 h with either the 10-Hz continuous or 100-Hz/30 s stimulation patterns did not potentiate the dephosphorylation of MEF2D (Fig. 6A, bottom), NFATc1 or MEF2A (data not shown). Likewise, none of the stimulation paradigms induced detectable expression of the MEF2 reporter in cross-sections this muscle (Fig. 6E), suggesting the already high calcineurin activity in these cells may render them refractory to relatively modest amounts of additional nerve activity. Taken together, these data further substantiate that the calcineurin pool in the largest and least active cells is relatively more sensitive to increases in contractive activity evoked by these paradigms (1 h and 200 s, respectively) largely exceeds the mean activity per hour of FF motor units (1.4–8.0 s/h; Ref 2) and associated IIb fibers, these data thus suggest that calcineurin-MEF2D signaling in fibers expressing this MHC is initiated when the aggregate amount of activity exceeds normal levels or when the interval between activation bursts is sufficiently brief (≤ 1 min). In contrast to MEF2D findings, neither paradigm of stimulation was effective in dephosphorylating MEF2A or NFATc1 in the medial gastrocnemius muscle (Fig. 6A, bottom panels), even after several hours (data not shown), suggesting that MEF2D is relatively more sensitive to nerve-mediated dephosphorylation via calcineurin.

In contrast to findings in the medial gastrocnemius, nerve-mediated activation of the soleus for 1–2 h with either the 10-Hz continuous or 100-Hz/30 s stimulation patterns did not potentiate the dephosphorylation of MEF2D (Fig. 6A, bottom), NFATc1 or MEF2A (data not shown). Likewise, none of the stimulation paradigms induced detectable expression of the MEF2 reporter in cross-sections this muscle (Fig. 6E), suggesting the already high calcineurin activity in these cells may render them refractory to relatively modest amounts of additional nerve activity. Taken together, these data further substantiate that the calcineurin pool in the largest and least active cells is relatively more sensitive to increases in contractive activity evoked by these paradigms (1 h and 200 s, respectively) largely exceeds the mean activity per hour of FF motor units (1.4–8.0 s/h; Ref 2) and associated IIb fibers, these data thus suggest that calcineurin-MEF2D signaling in fibers expressing this MHC is initiated when the aggregate amount of activity exceeds normal levels or when the interval between activation bursts is sufficiently brief (≤ 1 min).
**Neural Modulation of Calcineurin Activity in Skeletal Muscle**

**Fig. 6. Nerve electrical stimulation induces a calcineurin-dependent dephosphorylation of MEF2D and expression of the MEF2 reporter in type IIb fibers.** A and B, Western blots of MEF2D, MEF2A, and NFATc1 in whole cell extracts of the mouse medial gastrocnemius (MG) or soleus (SOL) under normal weightbearing conditions (Control) or after 1 h of 10-Hz continuous or intermittent 100-Hz burst stimulation of the sciatic nerve, in the absence or presence of CsA. For the latter pattern, 100-Hz bursts were administered every 30 s (in A), 1 min, 2 min, 5 min, 10 min, 15 min, or 30 min (in B). Filled arrows and open arrows in A and B, respectively, indicate the dephosphorylated and phosphorylated form(s) of these proteins. Results in A and B are representative of at least three independent experiments. MEF2C served as a loading control. C–E, cross-sections of mouse medial gastrocnemius (C and D) or soleus (SOL) and plantaris (PL) (E) muscles of desMEF2 Tg mice stained for β-gal activity (C and E) or immunolabeled for IIb MHC (D). Note that β-gal-positive cells in C–E were typed as expressing IIb MHC.

**Discussion**

The purpose of the present study was to test the hypothesis that skeletal muscle calcineurin signaling pathways are sensitive to nerve-mediated activity. To this end, we investigated whether calcineurin is more active in highly recruited muscles under normal weightbearing conditions and whether signaling via its substrates NFATc1, MEF2A, and MEF2D is potentiated in a way that matched increased activation profiles of muscles subjected to functional OV. Moreover, to directly assess the involvement of the electrogenic stimulus in the activation of this phosphatase in skeletal muscle cells, we examined whether calcineurin signaling via these substrates would be countered by neuronal quiescence or enhanced in response to electrical stimulation of the sciatic nerve. Here, we confirm our hypothesis and show the extent of calcineurin dephosphorylation of NFATc1 and MEF2 to be positively correlated with muscle usage in normal weightbearing muscles. Moreover, this response was nerve activity-dependent, because it was countered by silencing sciatic nerve action potentials with TTX. We also establish that complete signaling of this phosphatase via MEF2A or MEF2D proteins (i.e. leading to detectable increases in MEF2 transcriptional function) largely occurs when nerve activation is increased above normal levels such as with OV, supporting our contention that calcineurin likely cooperates with other activity-linked signaling pathways to effect a response via these proteins (18). Interestingly, this triggering of MEF2 transcriptional function by calcineurin occurred in all fiber types in response to increased activity, but most readily in fibers that are normally the least active, suggesting that signaling via this phosphatase is also influenced by the activation history of the muscle cell. Taken together, these findings provide novel insight toward our understanding of the nerve activity-dependent modulation of calcineurin signaling in adult skeletal muscle cells in vivo.

**Calcineurin Dephosphorylation of NFATc1 and MEF2 Is Nerve Activity-Dependent and Correlated with Muscle Usage**—It is postulated that sustained Ca\(^{2+}\) elevations such as those that occur in slow muscle fibers in response to tonic firing of their slow motor nerve are conducive to the activation of calcineurin (7). It is also thought that this phosphatase is refractory to the brief Ca\(^{2+}\) transients that are evoked in fast muscle cells in response to phasic firing of their fast motor nerve (7). We provide evidence that validates this model, in part, by showing that under normal weightbearing conditions calcineurin is activated to a greater extent (i.e. more extensive dephosphorylation of NFATc1, MEF2A, and MEF2D) in the soleus, a muscle that is highly recruited (20) and displays 2-fold higher resting intracellular Ca\(^{2+}\) levels compared with less active hindlimb muscle counterparts (26). Moreover, we provide first time evidence that this phosphatase is activated in a nerve activity-dependent fashion, because the dephosphorylation of NFATc1 and MEF2 proteins in these hindlimb muscles was countered by silencing sciatic nerve action potentials with TTX. On the other hand, our finding of a subtle calcineurin-dependent dephosphorylation of these substrates in the predominantly fast twitch plantaris or medial gastrocnemius suggests that this enzyme is not completely refractory to the activity profiles of fast motor units. Indeed, given the dephosphorylation of NFATc1, MEF2A, and MEF2D was positively correlated with the extent of recruitment of these plantar flexors (i.e. soleus > plantaris > medial gastrocnemius) under normal weightbearing conditions (20, 21), we thus refine this model and propose that the daily aggregate amount of nerve-mediated muscle usage and not the fiber type per se, is the key factor influencing calcineurin activity. When these results are considered with findings that calcineurin-dependent dephosphorylation of NFATc1, MEF2A, and MEF2D is potentiated in plantaris muscles subjected to OV, they provide evidence that all three of these transcription factors are targets of calcineurin downstream of nerve activity.

**Muscle Activity above Native Levels Is Prerequisite for Full Activation of MEF2**—Previous studies of cultured neurons and skeletal myocytes and whole muscle tissues have shown that dephosphorylation of MEF2A by calcineurin may be associated with an increase in the transactivating function of this protein (11, 27). Our finding of an absence of MEF2 reporter gene expression in all normal weightbearing muscles including the
soleus, despite a clear calcineurin-dependent dephosphorylation of MEF2A and MEF2D, appear to be in sharp contrast to these previous findings. The fact that in the present study, MEF2 transcriptional activity was only observed in overloaded plantaris and soleus muscles, and in type IIb fibers that were electrically stimulated in amounts that presumably exceeded their native activity levels, suggest that: 1) under normal weightbearing conditions, there exist molecular mechanisms that keep MEF2 from being fully active and, 2) an increase in nerve-mediated activity initiates signaling events that either relieve this repression or fully enhance the transcriptional function of MEF2. Indeed, recent converging lines of evidence suggest that calcineurin cooperates in a synergistic fashion with Ca\(^{2+}\)/CaM-dependent kinases (CaMKs) to activate MEF2 proteins in skeletal and cardiac muscle cells (11, 28). Activation of CaMKs appears to permit MEF2 transcriptional function by promoting the dissociation of the class II histone deacetylases from the DNA binding domain of MEF2 proteins (29, 30). In light of these results and our finding of an absence (this study) or a low amount (~15% of solei examined; Ref. 11) of MEF2 reporter gene expression in the normal weightbearing soleus of desMEF2 Tg mice, it is tempting to speculate that soleus contractile activity levels, although able to activate calcineurin, may just be below the threshold for CaMK activation. Although the present study did not address whether increased muscle activation above normal is required for complete signaling of calcineurin via NFATc1, previous findings that overexpression of both calcineurin and CaMK IV are required for full activation of NFAT1 in T-lymphocytes (31) suggests that this may indeed be the case.

It is proposed that frequent muscle fiber activation and contractile loading activate calcineurin signaling pathways, which lead to fiber hypertrophy and the expression of a slower contractile protein phenotype (15, 18). Our finding of negligible amounts of calcineurin-MEF2 signaling in the normal weightbearing soleus (this study and Ref. 11) coincides with previous reports that this phosphatase has a minor influence on the phenotype of this muscle in that CsA only induces subtle transitions in MHC expression after long term administration of this drug (7, 16). Similarly, our observation of a prominent induction of MEF2 reporter gene activity in both overloaded plantaris and soleus fibers concurs with our previous findings that calcineurin-dependent pathways have a profound effect on the size and MHC phenotype of skeletal muscle cells during compensatory growth (15, 18). Collectively, this suggests that expression of the MEF2 reporter is much more effective than the phosphorylation status of MEF2 or NFATc1 proteins as an indicator of the degree to which calcineurin signaling pathways are impacting muscle fiber phenotype. Reporter gene data thus provide additional evidence of an involvement of MEF2A or MEF2D proteins in the signaling of fiber hypertrophy and fast-to-slow fiber type transformations in response to OV (11, 18).

The dephosphorylation and induced transactivational activity of MEF2D observed in the plantaris in response to OV was likely nerve activity-dependent, because it followed closely the reported increase in EMG activity that occurs in this muscle under similar conditions (17). On the other hand, the MEF2A response to OV was more complex and also involved a transient increase in the expression of this protein at 5–7 days that could not be totally explained by the timing of the increase in muscle recruitment levels (17). The fact that this latter response was unique to OV muscles, and was only partially prevented with CsA, suggests that other parallel signaling pathways, active in the early phase of muscle growth, may cooperate with calcineurin to amplify signaling via MEF2A. Indeed, several lines of evidence suggest that insulin-like growth factor (IGF)-1 may act as a parallel signaling effector of MEF2A and an inducer of muscle growth. First, muscle IGF-1 levels increase in vivo in a transient fashion in response to OV (32), the timing of which just precedes the observed increase in MEF2A transcriptional function. Moreover, application of IGF-1 to cultured skeletal myocytes increases intracellular Ca\(^{2+}\) and activates calcineurin (33) and CaMK (30), both purported to induce hypertrophy of these cells, in part, via activation of MEF2 (30). The apparent requirement of accessory signaling events for the induction of MEF2 transcriptional function in skeletal muscles coincides with our previous demonstrations, and those of others, that activation of calcineurin is required, but alone not sufficient, to induce muscle fiber hypertrophy (18, 34) or fast-to-slow fiber type conversions (18, 35).

Fast Fiber Calcineurin Pool Appears Preferentially Sensitive to Nerve-mediated Activity—Muscle intracellular Ca\(^{2+}\) transients evoked by fast nerve activity were originally proposed to be unsuited for calcineurin activation (7). In contrast to this notion, we show that calcineurin signaling via MEF2 proteins is actually initiated most readily in the fastest, least active fibers (those expressing IIX or IIb MHC) in response to OV or electrical stimulation. Given the apparent requirement of increased activation for full induction of the MEF2 reporter, the ready initiation of calcineurin-MEF2 signaling in IIX and IIb fibers may relate to the fact that these cells are normally recruited for only seconds per day (2) and thus would respond quickly to what would appear to be a minimal foreign activity stimulus, but for these cells would be a substantial increase from native activity profiles. In contrast, slow twitch fibers are recruited for a large portion of the day (22–33% of daily time; Ref. 2) and would require more of a deviation from their native activity levels to initiate such signaling events. This is consistent with the finding that soleus MEF2 reporter expression was refractory to all electrical stimulation paradigms. Alternatively, the calcineurin pool in IIX and IIb fibers may be comparatively more sensitive to modulation by motor nerve activity. Indeed, several lines of evidence support this notion. For one, the catalytic subunit of calcineurin is expressed at comparatively higher levels in fast twitch muscles (35), and thus may convey greater signaling potency to fast fibers. In addition, transcript levels of myocyte-enhanced calcineurin interacting protein I and II, proteins that inhibit calcineurin signaling in muscle cells, are preferentially expressed at lower levels in fast compared with slow twitch skeletal muscles (36), thereby potentially lowering the threshold for calcineurin activation in the former cell types. Finally, greater responsiveness of IIX or IIb fibers to activation via the calcineurin-MEF2 pathway is also consistent with reports that these cells are the most sensitive and the first to adapt to increased nerve-mediated activity (3, 15, 37).

Frequent Nerve-mediated Depolarization of Muscle Cells Is Required for Calcineurin Activation—To elucidate which aspect of the electrogenic stimulus is important in activating calcineurin in hindlimb muscle cells, we compared the efficacy of various exogenous nerve electrical stimulation paradigms in the induction of calcineurin-dependent dephosphorylation of NFAT and MEF2 proteins. The 10–Hz continuous paradigm that we used mimicked the pulse frequency and tonic nature of slow motor units (2), whereas the intermittent 100–Hz burst stimulation paradigm mimicked the pulse frequency native to faster (fast fatigue-resistant and fast fatigable) motor unit types (2). Although the aggregate activity associated with 1–2 h of 10–Hz continuous electrical stimulation was well below levels required to initiate calcineurin-MEF2D signaling in the soleus, it was sufficient to activate this pathway in plantaris...
and medial gastrocnemius IIb fibers. The effectiveness of 10-Hz continuous stimulation in activating calcineurin in these fastest fibers is consistent with previous findings that this paradigm elicits a sustained elevation of muscle Ca\(^{2+}\) (0.15–0.3 \(\mu\)M) (8) toward levels known to initiate calcineurin signaling in B-lymphocytes (38). Interestingly, we found that an intermittent 100-Hz stimulus pattern was just as effective as the 10-Hz continuous paradigm in inducing a calcineurin-dependent dephosphorylation of MEF2D when the interburst interval was sufficiently brief (<1 min), suggesting that the aggregate amount of activity or the integral of the Ca\(^{2+}\) signal are key to activating calcineurin, rather than the pulse frequency or associated amplitude of the Ca\(^{2+}\) transient. Nonetheless, the finding that MEF2 reporter gene was not induced in slower type I, IIa, or IIx fiber types by any of these stimulation paradigms supports the contention that muscle activation above native levels is required to induce a significant increase in calcineurin signaling via MEF2 proteins.

Calcineurin Substrates Are Differentially Sensitive to Calcineurin Activation—Another interesting finding of this study was that NFATc1 and MEF2A were less readily dephosphorylated compared with MEF2D in response to calcineurin activation. One possible explanation for the difference in the sensitivity of MEF2 and NFATc1 proteins is that MEF2 resides in the nucleus of muscle cells (39) and thus would be exposed to a different Ca\(^{2+}\) milieu compared with cytoplasmic localized NFATc1 (25). Indeed, during excitation-contraction coupling, the Ca\(^{2+}\) transients evoked in the nucleus of muscle cells are more sustained (i.e. have slower rise and decay times) compared with cytoplasmic levels (40), thereby promoting calcineurin activation. Nonetheless, the fact that dephosphorylation of MEF2D was more complete than MEF2A in the OV plantaris suggests that this protein may play a role in the establishment of the new myofiber phenotype. Although we did show muscle regeneration to potentiate the dephosphorylation of MEF2A and MEF2D in the plantaris, MEF2 reporter gene expression was not detected in nascent fibers in cross-sections of the plantaris or remnant portions of the regenerating gastrocnemius in OV mice, suggesting that calcineurin-MEF2 signaling may play a comparatively minor role at this stage of myogenesis.

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Nerve Activity-dependent Modulation of Calcineurin Signaling in Adult Fast and Slow Skeletal Muscle Fibers
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