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Muscle Fiber Type Specific Induction of Slow Myosin Heavy Chain 2 Gene Expression by Electrical Stimulation

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Abstract

Vertebrate skeletal muscle fiber types are defined by a broad array of differentially expressed contractile and metabolic protein genes. The mechanisms that establish and maintain these different fiber types vary throughout development and with changing functional demand. Chicken skeletal muscle fibers can be generally categorized as fast and fast/slow based on expression of the slow myosin heavy chain 2 (MyHC2) gene in fast/slow muscle fibers. To investigate the cellular and molecular mechanisms that control fiber type formation in secondary or fetal muscle fibers, myoblasts from the fast pectoralis major (PM) and fast/slow medial adductor (MA) muscles were isolated, allowed to differentiate in vitro, and electrically stimulated. MA muscle fibers were induced to express the slow MyHC2 gene by electrical stimulation, whereas PM muscle fibers did not express the slow MyHC2 gene under identical stimulation conditions. However, PM muscle fibers did express the slow MyHC2 gene when electrical stimulation was combined with inhibition of inositol triphosphate receptor (IP3R) activity. Electrical stimulation was sufficient to increase nuclear localization of expressed Nuclear-Factor-of-Activated-T-cells (NFAT), NFAT-mediated transcription, and slow MyHC2 promoter activity in MA muscle fibers. In contrast, both electrical stimulation and inhibitors of IP3R activity were required for these effects in PM muscle fibers. Electrical stimulation also increased levels of peroxisome-proliferator-activated receptor- γ co-activator-1 (PGC-1 α) protein in PM and MA muscle fibers. These results indicate that MA muscle fibers can be induced by electrical stimulation to express the slow MyHC2 gene and that fast PM muscle fibers are refractory to stimulation-induced slow MyHC2 gene expression due to fast PM muscle fiber specific cellular mechanisms involving IP3R activity.

Keywords

stimulation; NFAT; IP3R; fiber-type; myosin

Introduction

Vertebrate skeletal muscles contain muscle fibers that vary in their contraction and relaxation rates and metabolic capacities. Fast and slow contracting muscle fibers express contractile and

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metabolic genes that impart these physiological characteristics of fast and slow fibers. In avian species, expression of the slow myosin heavy chain (MyHC) 2 gene most clearly distinguishes fast versus slow muscle fiber types. All avian muscle fibers (except some fibers of the adult anterior latissimus dorsi [1–3]) express a fast MyHC isoform. In addition to fast MyHC(s), most adult slow muscle fibers also express slow MyHC2. For example, the adult pectoralis major (PM) contains muscle fibers that express fast MyHC genes and no slow MyHC genes. In contrast, all of the muscle fibers of the adult medial adductor (MA) muscle express both fast MyHC and slow MyHC2 genes [1].

The mechanisms that control skeletal muscle fiber phenotype can be broadly classified as intrinsic and extrinsic. Intrinsic control of muscle fiber type identity is evident in embryonic avian development of primary muscle fibers. Fast and slow primary muscle fibers arise both in vitro and in vivo from distinct fast and slow myogenic cell lineages [4–6]. Clonal analysis of isolated embryonic myoblasts demonstrated that single myoblasts contained inherent developmental programming that resulted in differentiation of myoblast-specific, distinct muscle fiber types [4]. This analysis was extended into an in vivo experimental model system in which embryonic myoblasts transplanted into embryonic limbs differentiated into muscle fibers that were identical to muscle fibers formed in vitro with respect to fast and slow MyHC gene expression [6]. Patterns of innervation and denervation do not significantly alter the pattern of MyHC gene expression in these primary fibers [7,8].

In contrast to intrinsic control of primary fiber type development, extrinsic factors such as innervation significantly affect patterns of MyHC gene expression in secondary or fetal muscle fibers [9]. All secondary avian muscle fibers in vitro and in vivo express a fast MyHC isoform. We have previously shown that expression of the slow MyHC2 gene and fiber type is regulated by innervation [10]. In vivo, slow MyHC2 gene expression is diminished by administration of curare to block neuromuscular transmission [7,8]. In vitro, innervation of muscle fibers induced slow MyHC2 gene expression. However, this inductive event was dependent on the intrinsic nature of the innervated muscle fibers themselves. Muscle fibers formed from myoblasts isolated from the slow MA muscle expressed slow MyHC2 when innervated. However, muscle fibers formed from myoblasts isolated from the fast PM muscle were refractory to innervation-induced expression of the slow MyHC2 gene. Administration of curare also blocked slow MyHC2 gene expression in innervated MA muscle fibers in vitro [10].

We have previously shown that slow MyHC2 gene expression in innervated muscle fibers is regulated by a signal transduction cascade that involves ryanodine receptor 1 (RyR1) and inositol triphosphate receptor (IP₃R1) activities [11,12]. These calcium release channels control intracellular calcium fluxes following neural stimulation [13]. Interestingly, slow MyHC2 gene expression in innervated fast PM muscle fibers appears to be repressed by muscarinic acetylcholine receptor activity and downstream signaling via Gαq and protein kinase C [14]. Inhibition of these signaling components in innervated fast PM muscle fibers causes a fiber type transition by expression of the slow MyHC2 gene. Therefore, PM and MA muscle fibers display inherent differences in their response to innervation stimuli as reflected in their differential signaling pathways leading to expression of the slow MyHC2 gene.

Several transcriptional regulators have been associated with expression of slow fiber type specific genes. The nuclear factor of activated T cells (NFAT) is a target of the calcium/calmodulin-dependent protein phosphatase, calcineurin. Dephosphorylation of NFAT by calcineurin permits nuclear accumulation of NFAT and subsequent NFAT-mediated transcriptional regulation in a variety of cell types, including skeletal muscle fibers [15–17]. NFAT has been implicated in the calcineurin-dependent regulation of the slow troponin I and myoglobin genes as well as the mammalian slow MyHC gene [18,19]. The avian slow MyHC2 gene is also regulated by NFAT binding sites within the slow MyHC2 promoter [20]. Myocyte

enhancer factor 2 (MEF2) also has been implicated in the calcineurin and activity-dependent regulation of a variety of muscle specific genes including troponin I and MyHC genes [21]. MEF2 also regulates the avian slow MyHC2 gene [20].

PGC-1 α is a transcriptional co-activator, initially identified in a brown fat cell line [22] and subsequently shown to be an important regulator of the adaptive responses of skeletal muscle to exercise and activity. PGC-1 α gene expression occurs preferentially in slow twitch, oxidative muscle fibers compared to fast twitch, glycolytic muscle fibers [23,24]. PGC-1 α activity is most notably associated with increased mitochondrial biogenesis and oxidative metabolism in PGC-1 α overexpressing transgenic mice and in exercised muscle [23–26]. Exercise-induced PGC-1 α gene expression occurs via activation of the p38 MAPK pathway [27]. PGC-1 α muscle-specific knockout mice displayed reduced exercise capacity, increased exercise-induced muscle fiber damage, and a moderate reduction in slow type I muscle fibers [28].

Previous studies of motor neuron development and innervation of muscle fiber types by “fast” and “slow” motor neurons has indicated that the motor neurons develop much of their distinct physiological properties before muscle fiber innervation [29–31]. Our previous studies using motor neurons to stimulate muscle fibers in vitro did not characterize the physiological properties and firing rates of individual motor neurons. Therefore, it was possible that selective motor neuron-muscle fiber type interactions occurred in the nerve-muscle co-cultures facilitating slow MyHC2 gene expression in MA muscle fiber cultures contacted only by “slow” motor neurons. To further elucidate the mechanism of fiber type control by expression of the slow MyHC2 gene and determine whether direct electrical stimulation of muscle fibers can recapitulate the innervation-dependent and fiber type restriction of slow MyHC2 gene expression, muscle fibers were directly stimulated by electrical pulses instead of motor neuron innervation. Direct electrical stimulation induced PGC-1 α gene expression, fiber type specific expression of the slow MyHC2 gene, and this latter expression was dependent upon NFAT and IP3R activity.

Materials and Methods

Cell culture

Myoblasts from the pectoralis major (PM) and medial adductor (MA) muscles of embryonic day 13 (ED13) chick embryos were isolated as previously described [32]. Cells were plated onto collagen coated Thermanox coverslips (Nunc) and placed into four-well rectangular plates (Nunc). Cells were grown in Ham's F-10 medium supplemented with 10% horse serum (Hyclone), 5% chick embryo extract, 1.1mM CaCl₂, 2mM glutamine, and antibiotic (penicillin/streptomycin/amphotericin B (Gibco)). Medium was replaced every other day before electrical stimulation and every day during electrical stimulation. For some cultures, inhibitors of inositol triphosphate receptor (IP3R), 2-aminoethoxyborate (2-APB) or xestospongin D, were added to cell culture to final concentrations of 100uM or 10uM, respectively, one-half hour before electrical stimulation and thereafter for the remainder of the cell culture period.

Electrical stimulation

The stimulation protocol was optimized for expression of the slow MyHC2 gene as determined by western blotting. Muscle fibers were repeatedly stimulated for 30 minutes with 20–50V at 1–20Hz for 4 days in a biphasic wavefront pattern using a Hugo Sachs Stimulator CS Type 223 controller (Harvard Apparatus). Each 30-minute stimulation period was followed by a 15-minute rest period.

DNA constructs and transfections

The expression construct, pSH160c, containing the human NFATc1 cDNA preceded by the FLAG epitope tag was generously provided by G. Crabtree [33]. NFATLuc and MEF2Luc transcription factor sensor constructs were generously provided by E. Olson (UT Southwestern). The SRELuc sensor was obtained from Panomics. The 2279bp slow MyHC2 promoter linked to the firefly luciferase gene is contained within 2279SM2Luc which has been previously described and characterized. 2279SM2Luc containing the NFAT binding site mutation has also been described and characterized [20]. Transfection and promoter activity control DNAs, pGL3Basic and pRL-SV40, were obtained from Promega. All DNA transfections into PM and MA myoblasts used 1 μ g DNA/35mm culture dish and included Lipofectamine 2000 (Invitrogen).

Promoter activity assays

Firefly luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega) and were normalized for variation in transfection efficiencies by determination of Renilla luciferase activities from transfection of pRL-SV40. Background luciferase activities were determined in extracts from cells transfected with the promoterless pGL3Basic vector DNA.

Western blotting

For western blots of MyHCs and actin, whole cell lysates of cultured cells were prepared on day seven of incubation. Coverslips with cells were washed three times with phosphate-buffered saline (PBS), drained, and allowed to remain on ice for 15 minutes under 70 μ l of RIPA buffer (150mM NaCl, 1% NP-40, 0.05% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris, pH 7.5) containing protease inhibitors (Complete Mini, Roche Diagnostics). Cells were scraped from the coverslips and homogenized by 20 rapid passages through a 25-gauge needle. For western blots of PGC-1 α and actin, coverslips were washed with PBS, drained, and cells were scraped off the coverslips in 1ml cold PBS. These cells were pelleted by centrifugation at 2,000 $\times$ g for 2 minutes at 4°C in an Eppendorf centrifuge. Cells were homogenized as above in 200 μ l of 20mM Tris-HCl, 1mM EDTA, 25mM NaCl, 0.5% Triton X-100, pH. 7.5. The extracts were incubated on ice for 1 hour and then cleared by centrifugation at 20,000 $\times$ g for 10 minutes at 4°C in an Eppendorf centrifuge. Protein concentrations were determined using a BCA protein assay reagent (Pierce). Equal amounts of protein were denatured for 5 minutes at 95°C in SDS-PAGE sample buffer, electrophoresed in SDS polyacrylamide gels, and transferred to nitrocellulose membranes. Blots were placed in blocking solution (5% non-fat dry milk, 0.05% Tween-20 in PBS) overnight. Supernatants of monoclonal antibody F59 and S58 that specifically recognize fast MyHCs and slow MyHC2, respectively, were diluted 1:10 in blocking solution. The specificities of these antibodies have been previously characterized [7]. Anti-sarcomeric actin antibody (Sigma) and PGC-1 α antibody (BD Biosciences) were diluted 1:2000 and 1:1000, respectively, in blocking solution. Diluted antibodies were incubated with the blots for 1 hour at room temperature. Blots were washed three times with PBS containing 0.05% Tween-20. The primary antibodies were recognized by horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz Biotech) diluted 1:1000 in blocking solution. Secondary antibodies were incubated with the blots for 1 hour at room temperature and then washed three times as before. Blots were developed by chemiluminescent detection of HRP (GE Lifesciences).

Immunocytochemistry

Muscle fibers were washed three times with PBS and then fixed with 100% methanol for 10 minutes. The cells were washed again with PBS and then incubated in blocking solution, consisting of 5% horse serum, 2% bovine serum albumin in PBS, for 1 hour at room

temperature. F59 and S58 monoclonal antibodies that recognize fast MyHC and slow MyHC2, respectively [7] were diluted 1:10 in blocking solution. The anti-FLAG epitope antibody (Sigma) was diluted 1:4000 in blocking solution. Antibodies were applied to the cells for 1 hour at room temperature and subsequently washed away with PBS three times. Primary antibodies were detected with fluorochrome-conjugated secondary antibodies (Vector Labs), diluted 1:100 in blocking solution, and applied to the cells for 1 hour at room temperature. Nuclei were detected by incubation of cells in 1.2uM DAPI in PBS for 10 minutes. Muscle fibers were washed with PBS as before, and coverslips were applied for viewing by fluorescent microscopy.

Results

Stimulation-induced slow MyHC2 gene expression

Slow MyHC2 gene expression in avian secondary muscle fibers is elicited by functional innervation in vivo and in vitro [9]. Furthermore, muscle fiber-nerve co-culture studies suggested that innervation-induced expression of the slow MyHC2 gene was restricted to muscle fibers formed from myoblasts of slow muscle origin [10]. To determine whether the restriction of slow MyHC2 gene expression to particular secondary muscle fibers is inherent to the muscle fibers themselves, functional innervation was replaced by direct electrical stimulation. Myoblasts were isolated from ED13 PM and MA muscles and allowed to differentiate into muscle fibers. These fibers were then electrically stimulated using a variety of stimulation patterns. Expression of the slow MyHC2 gene was then analyzed by western blotting and immunocytochemistry using the slow MyHC2-specific monoclonal antibody, S58 (Fig. 1).

Slow MyHC2 gene expression was induced by direct electrical stimulation specifically in MA muscle fibers. This expression was maximal with a repetitive stimulation pattern of 10Hz at 50V for 30 minutes followed by a 15-minute rest period (Fig. 1A). Each protein extract and all PM and MA muscle fibers (Fig. 1B) contained a fast MyHC isoform as determined by immunodetection with the F59 monoclonal antibody. Importantly, expression of the slow MyHC2 gene was restricted to MA muscle fibers. Muscle fibers derived from myoblasts of fast muscle (PM) origin did not express the slow MyHC2 gene under any stimulation protocol tested. These results indicate that the capacity to express the slow MyHC2 gene is restricted to muscle fibers of fast/slow muscle origin (e.g. MA), not fast muscle origin, and that this restriction is based in the muscle fibers themselves.

Stimulation-induced increase of PGC-1 α protein in both PM and MA muscle fibers

Since direct electrical stimulation of muscle fibers in vitro induced slow MyHC2 gene expression in MA muscles, we hypothesized that other genes characteristic of the slow muscle fiber phenotype were also induced. Therefore, expression of the gene encoding the transcriptional co-activator, PGC-1 α , was assessed by western blotting of protein extracts from ED13 PM and MA muscles in vivo and from PM and MA muscle fiber cultures with and without electrical stimulation (Figure 2). PGC-1 α was chosen for analysis of proteins characteristic of slow muscle fibers because it is a key regulator of mitochondrial biogenesis and therefore of metabolic capacities of slow muscle fibers [23]. Its role is distinct from the contractile properties conferred by expression of the slow MyHC2 gene, yet still reflects the slow muscle fiber phenotype. PGC-1 α was more abundant in ED13 MA muscle extracts compared to the PM muscle extracts. In non-stimulated PM and MA muscle fiber cultures, PGC-1 α was detectable, but at significantly reduced levels compared to in vivo MA muscle extracts. However, stimulation of MA muscle fibers in vitro with the same stimulation regime that elicited slow MyHC2 gene expression also increased PGC-1 α protein levels comparable to the protein levels in MA muscle in vivo. Interestingly, the stimulation regime also increased

PGC-1 α protein levels in PM muscle fiber cultures. These levels were also comparable to both the MA levels in vivo and in vitro. Therefore, direct electrical stimulation increased PGC-1 α gene expression in both PM and MA muscle fiber cultures.

Inhibition of IP3R activity de-represses slow MyHC2 gene expression in stimulated fast muscle fibers

To determine whether IP3R activity in muscle fibers contributed to the fiber type specific expression of the slow MyHC2 gene, IP3R activity was pharmacologically inhibited with two distinct compounds. Stimulated and non-stimulated PM and MA muscle fibers were incubated in medium containing either 100 μ M 2-APB or 10 μ M xestospongine D. Muscle fibers were then immunostained with F59 and S58 monoclonal antibodies (Fig. 3). All avian muscle fibers, whether of PM or MA origin, immunostained for fast MyHC with monoclonal antibody F59. Non-stimulated PM and MA muscle fibers in the presence of 2-APB or xestospongine D expressed only a fast MyHC isoform and did not express the slow MyHC2 gene. Also, addition of 2-APB or xestospongine D versus control medium did not alter the positive immunostaining of stimulated MA muscle fibers for slow MyHC2 (Figs. 1 and 3). However, slow MyHC2 was detected in stimulated PM muscle fibers cultured in medium containing 2-APB or xestospongine D. These results indicate that IP3R activity represses slow MyHC2 gene expression in stimulated fast muscle fibers.

Localization of NFAT in stimulated muscle fibers

Since several slow muscle fiber type specific genes are regulated by NFAT [18,19] which is itself indirectly controlled by intracellular calcium transients such as those elicited by IP3R1 activity [34], NFAT nuclear localization was determined in stimulated and non-stimulated PM and MA muscle fibers with and without the IP3R inhibitor, 2-APB. PM and MA myoblasts were transfected with an NFATc1 expression vector tagged with a FLAG epitope tag [33] and allowed to differentiate into muscle fibers. At the end of the stimulation period, muscle fibers were immunostained with monoclonal antibody F59 and a FLAG epitope tag antibody (Fig. 4). In MA muscle fibers, NFATc1-FLAG localized to muscle fiber nuclei only when these fibers were electrically stimulated. The presence of 2-APB in the culture medium did not appear to have a significant effect on NFAT localization in these muscle fibers. Interestingly, PM muscle fibers displayed a different localization pattern of NFATc1-FLAG. Whereas NFATc1-FLAG was nuclear localized in stimulated MA muscle fibers in control medium, no evidence for nuclear localization in stimulated PM muscle fibers in control medium was observed. However, NFATc1-FLAG was nuclear localized in stimulated PM muscle fibers incubated in medium containing 100 μ M 2-APB. Therefore, NFATc1 nuclear localization is regulated in a muscle fiber type specific manner and is dependent upon IP3R activity in fast PM muscle fibers.

Effects of stimulation and 2-APB on transcription regulator activities

Both NFAT and MEF2 regulate slow MyHC2 promoter activity in secondary muscle fibers [20]. To determine the effects of electrical stimulation and inhibition of IP3R on activities of the transcriptional regulators NFAT and MEF2, transcriptional DNA sensors containing the luciferase gene and regulated by multiple NFAT and MEF2 binding sites, respectively, were transfected into ED13 PM and MA myoblasts. After differentiation, stimulated and non-stimulated muscle fibers were incubated in medium with and without 2-APB. NFAT-mediated transcription demonstrated responses to both stimulation and IP3R inhibition (Fig. 5A). In PM muscle fibers, stimulation alone did not significantly activate NFAT-mediated transcription. The presence of 2-APB also did not activate NFAT-mediated transcription in non-stimulated PM muscle fibers. However, the combination of stimulation and 2-APB increased NFAT sensor activity approximately 6 fold in PM muscle fibers. In contrast to the effects on PM

muscle fibers, stimulation and the presence of 2-APB, individually and in combination, increased NFAT sensor activity in MA muscle fibers. The increased NFAT sensor activity in stimulated MA muscle fibers was significantly greater than (approximately twice) the activity in non-stimulated MA muscle fibers cultured in medium with 2-APB. These results are in general agreement with the NFAT nuclear localization results in Fig. 4, indicating that NFAT-mediated transcription occurs in cells when NFAT is nuclear localized. The same conditions that significantly increased NFAT-mediated transcription also induced slow MyHC2 gene expression (Figs. 1 and 3).

MEF2-mediated transcription was also significantly increased by electrical stimulation of MA muscle fibers (Fig. 5B). Luciferase activity from stimulated MA muscle fibers was significantly greater than (4–5 times) activity in non-stimulated PM or MA muscle fibers. However, no other condition (stimulation and/or IP3R inhibition) affected MEF2-mediated transcription. Lastly, serum response element (SRE)-based transcription in PM and MA muscle fibers was not significantly affected by any of the tested conditions (Fig. 5C).

Stimulation-induced activation of the slow MyHC2 promoter

To determine the direct effects of electrical stimulation and IP3R activity on regulation of the slow MyHC2 promoter, 2279SM2Luc was transfected into myoblasts from PM and MA muscles. These myoblasts were allowed to differentiate and were then electrically stimulated as before. This slow MyHC2 promoter is activated in a manner identical to the endogenous slow MyHC2 promoter and is activated by NFAT and MEF2 cis-regulatory sites [20]. Relative luciferase activities were measured (Fig. 6A). Slow MyHC2 promoter activity in MA muscle fibers was significantly increased by electrical stimulation with and without the addition of 2-APB to the cell culture medium. In contrast, slow MyHC2 promoter activity in PM muscle fibers was not significantly increased by electrical stimulation alone. Also, addition of 2-APB to the cell culture medium did not affect slow MyHC2 promoter activity in non-stimulated muscle fibers. Interestingly, stimulation of PM muscle fibers in the presence of 2-APB increased slow MyHC2 promoter activity to a relative level that was not significantly different than the activity in slow stimulated MA muscle fibers. These results indicate that fast PM muscle fibers are refractory to stimulation-induced activation of the slow MyHC2 promoter and that this repression can be abrogated by 2-APB.

To determine whether the NFAT binding site located within the slow MyHC2 promoter mediated the stimulation and 2-APB-induced activation of the promoter in PM and MA muscle fibers, activity of the slow MyHC2 promoter harboring a mutation of the NFAT binding site was measured (Fig. 6B). This site is functional and interacts with NFATc1 in innervated MA muscle fibers [20]. Mutation of the NFAT binding site blocked activation of the slow MyHC2 promoter in response to electrical stimulation and 2-APB.

Discussion

Skeletal muscle is comprised of distinct fiber types characterized by patterns of gene expression that define the contractile and metabolic properties of fast and slow muscle fibers. This fiber type specific gene regulation can be governed by extrinsic stimuli such as innervation or direct electrical stimulation. Fiber type specific genes may also be regulated by cellular mechanisms intrinsic to the muscle fibers themselves. Alternatively, the definitive muscle fiber phenotype may be controlled by adaptive coordination of both extrinsic and intrinsic regulatory mechanisms. In other words, external stimuli such as activity-induced innervation patterns may elicit muscle fiber type transitions that are restricted by intrinsic muscle fiber type specific mechanisms.

Abundant evidence exists for fiber type regulation by extrinsic factors such as innervation [9]. Denervation and cross-reinnervation studies have consistently demonstrated innervation-induced regulation of fiber type. It is also clear that the majority of muscle fibers affected by altered neural input are secondary or fetal muscle fibers [8,35]. This is perhaps expected since most vertebrate muscles are principally composed of secondary muscle fibers. In addition to evidence for extrinsic control of fiber type, it is clear that muscle fiber type identity can be specified by intrinsically regulated mechanisms. Individual clonal embryonic myoblasts exhibit stable commitment to differentiation of distinct muscle fiber types in vitro and in vivo [4,6]. The cell lineage based intrinsic control of muscle fiber type differentiation is most clearly evident in these embryonic myoblasts and the primary muscle fibers they form.

The experimental results reported here indicate that secondary muscle fiber type is regulated by a coordinated interplay of both extrinsic stimuli and intrinsic cellular mechanisms. Direct electrical stimulation of MA muscle fibers induced expression of the slow MyHC2 gene. These stimulated fast/slow MA muscle fibers in vitro reflect the MyHC genes expressed by MA muscle fibers in vivo [1]. Slow MyHC2 gene expression was induced by chronic stimulation of MA muscle fibers at 10Hz and 50V for 30 minutes with intervening 15 minute rest periods. Similar patterns of electrical stimulation elicited slow MyHC gene expression in rabbit muscle fibers in vitro [19]. Importantly, stimulation-induced expression of the slow MyHC2 gene was fiber type restricted. PM muscle fibers were refractory to stimulation-induced expression of the slow MyHC2 gene, indicating that intrinsic qualities of fast PM muscle fibers repressed a fast-to-slow fiber type transition. Of equal significance is the restriction of differential expression of the slow MyHC2 gene in fast PM versus fast/slow MA muscle fibers in the presence of electrical stimulation and the absence of cell-cell interactions with motor neurons. These results remove potentially confounding and unknown interactions between unspecified populations of motor neurons with specific PM and MA fiber types in nerve-muscle co-cultures.

Previous studies in various mammalian model systems have suggested the existence of myogenic cells intrinsically predisposed to specific fiber type formation during fetal myogenesis and in the adult. Electrical stimulation of myotubes derived from satellite cells of the rat soleus and tibialis anterior preferentially induced expression of slow type I MyHC in the soleus-derived myotubes [36]. These results were extended in analyses of myotubes derived from satellite cells from single fibers of mouse EDL and soleus muscles. Slow type I MyHC expression occurred predominantly in the myotubes derived from satellite cells of the soleus, indicating intrinsic diversity among satellite cells [37]. In addition, transplantation of rat myoblasts into developing limbs yielded fiber types indicative of multiple myogenic populations with developmental potentials restricted by both intrinsic and extrinsic factors [38]. Furthermore, in vivo studies of denervated and chronically stimulated rat EDL and soleus muscles indicated intrinsic differences in established muscle fibers with respect to the fiber type specific adaptive ranges of MyHC gene expression in response to different patterns of electrical stimulation [39]. Therefore, intrinsically defined restriction of myogenic cells to the formation of specific fiber types at multiple stages of myogenesis is a common theme in many vertebrate species.

PGC-1 α is a key transcriptional regulator of mitochondrial biosynthesis and the associated metabolic capacity of muscle fibers. Therefore, it was of interest to determine whether electrical stimulation of PM and MA muscle fibers affected PGC-1 α gene expression. In vivo, the slow MA muscle contained significantly more PGC-1 α compared to the fast PM muscle, and this preferential PGC-1 α gene expression in slow muscles is in agreement with previous studies [23]. Electrical stimulation increased PGC-1 α protein levels. Interestingly, the increase occurred in both slow MA and fast PM muscle fiber cultures, and the increased PGC-1 α protein levels were not significantly different between stimulated PM and MA muscle fibers. The

difference in PGC-1 α protein levels between fast PM muscle in vivo and the stimulated PM muscle fibers in vitro is likely due to the chronic, slow electrical stimulation regime in vitro. Interestingly, the similar increase of PGC-1 α in both PM and MA muscle fiber cultures in response to electrical stimulation was different than the MA muscle fiber specific increase in slow MyHC2 gene expression. The differences in response to stimulation may reflect different mechanistic, cell signaling responses to stimulation, leading to different modes of metabolic versus contractile protein gene expression. This differential mode of metabolic versus contractile protein gene expression (including PGC-1 α versus MyHC isoforms) in specific muscle fiber types has been previously indicated in studies of oxidative and glycolytic protein gene expression relative to muscle fiber type [40].

We have previously shown that IP3R1 activity repressed slow MyHC2 gene expression in innervated fast muscle fibers [10]. To determine whether this repression was centered on the muscle fibers themselves and not contributed by IP3R1 inhibition in the associated motor neurons, IP3R1 activity was inhibited in muscle fibers that were directly electrically stimulated. Inhibition of IP3R1 activity in electrically stimulated PM muscle fibers de-repressed slow MyHC2 gene expression. In contrast, electrical stimulation alone was sufficient to induce slow MyHC2 gene expression in MA muscle fibers. Therefore, IP3R1 activity contributes to the intrinsic fiber type specific response to electrical stimulation.

The slow MyHC2 promoter is partially controlled by two NFAT binding sites in its transcriptional regulatory region [20]. NFAT transcription factors are responsive to calcium-induced activation of the protein phosphatase, calcineurin, leading to activity dependent cytoplasmic to nuclear translocation and subsequent NFAT-mediated transcription [19,41–43]. In addition, IP3R activity can contribute to the regulation of NFAT-mediated transcription by regulation of its cellular localization [34]. In non-stimulated PM and MA muscle fibers, NFAT was not detected in nuclei by immunocytochemical staining. NFAT was detected in nuclei of stimulated MA fibers. In PM muscle fibers, NFAT was nuclear localized only when both electrical stimulation and an inhibitor of IP3R activity were applied. This stimulation and IP3R activity dependent nuclear localization of NFAT reflected the pattern of slow MyHC2 gene expression in both MA and PM muscle fibers.

This pattern of fiber type specific NFAT regulation was also observed in NFAT-mediated transcription. Stimulated MA muscle fibers and PM muscle fibers that were both stimulated and treated with 2-APB exhibited significantly increased NFAT-mediated transcription. Slow MyHC2 promoter activity in both MA and PM muscle fibers also reflected the stimulation and IP3R1-dependent regulation of NFAT nuclear localization and transcriptional activity.

Recent studies of activity-dependent nucleo-cytoplasmic shuttling of NFAT isoforms and subsequent regulation of MyHC gene expression have shown that specific NFAT isoforms are differentially regulated by electrical stimulation patterns indicative of fast and slow muscle fibers [44]. Whereas all four NFAT isoforms (NFATc1, c2, c3, and c4) translocated to nuclei in muscle fibers with a slow fiber stimulation pattern, only NFATc3, NFATc4, and to a lesser extent NFATc2, translocated to nuclei in muscle fibers with a fast fiber stimulation pattern. Therefore, NFATc1 appears to be preferentially regulated by a slow fiber stimulation pattern.

Results presented here also demonstrate that NFATc1 is differentially regulated by electrical stimulation in a fiber type specific manner. Since it is proposed that NFAT nuclear localization is controlled by the calcium-dependent phosphatase, calcineurin, [45] and that IP3R activity controls NFAT localization as well, it is intriguing to hypothesize that fiber type specific IP3R activity may control calcineurin activity which, in turn, controls NFAT nuclear localization. Further studies of the mechanism of fiber type specific regulation of NFAT localization are needed to address this hypothesis.

Skeletal muscle cells can exhibit significant adaptive responses to activity, innervation, and direct electrical stimulation. These adaptive responses often include changes in contractile protein gene expression to establish and maintain the new contractile properties. This is evident in the fast-to-slow muscle fiber type transition of chronically stimulated MA muscle fibers. These muscle fibers express the slow MyHC2 gene in response to electrical stimulation. Although these adaptive responses can be significant, there is variability in the extent to which different muscle fibers will respond to particular external stimuli. For example, PM muscle fibers were refractory to expression of the slow MyHC2 gene under stimulation conditions that induced expression of this gene in MA muscle fibers. Therefore, the subset of contractile protein genes that can be expressed due to external stimuli appears to be intrinsically regulated in a fiber type specific manner. In a PM muscle fiber, the intrinsic restriction of its response to chronic low frequency stimulation appears to be dependent on IP3R1 activity and its downstream effects on transcriptional regulators such as NFAT that control fiber type specific gene expression.

Other mechanisms of transcriptional regulation have been implicated in muscle fiber type specific gene expression of MyHC genes. Several reports have shown that the transcriptional regulator Sox6 represses slow MyHC gene expression in both mice and zebrafish [46,47]. Furthermore, PRDM1 promotes slow muscle fiber development by repression of the Sox6 transcriptional repressor in zebrafish [47]. Further studies illustrate the need to carefully distinguish the mechanisms of fiber type development in primary versus secondary myogenesis. Whereas Sox6 transcriptional repression of slow MyHC gene expression in mouse fast muscle fibers occurs during fetal (secondary) myogenesis, the role of PRDM1 repression of Sox6 activity appears to be more prevalent during zebrafish adaxial muscle fiber development with only a subset of secondary slow fibers requiring PRDM1 activity [48]. Nevertheless, these studies and the results reported here demonstrate that both fast and slow muscle fiber type development likely involve multiple levels of activation and repression of fiber type specific genes throughout myogenesis.

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Figure 1A

Figure 1B **Fig. 1.**

Immunodetection of slow MyHC2 in electrically stimulated muscle fibers. PM and MA muscle fibers were electrically stimulated and fast MyHC and slow MyHC2 were detected using monoclonal antibodies F59 and S58, respectively. A) Western blot analysis of protein extracts from non-stimulated PM and MA muscle fibers and from PM and MA muscle fibers treated with the indicated electrical stimulation patterns. B) Immunocytochemical detection of fast MyHC and slow MyHC2 in non-stimulated (-Stim) PM and MA muscle fibers and in muscle fibers stimulated (+Stim) with 50V at 10Hz.

Figure 2A

Figure 2B

Fig. 2.

Western blot analysis of PGC-1 α gene expression. A) PGC-1 α and sarcomeric actin were detected in western blots of protein extracts from ED13 chick PM and MA muscles (lanes 1 and 2), from PM and MA muscle fiber cultures without electrical stimulation (lanes 3 and 4), and from stimulated PM and MA muscle fibers (lanes 5 and 6). B) Quantification of the relative

abundance of PGC-1 α protein after normalization using the abundance of sarcomeric actin. Values are means $\pm$ SE, n = 4. *P < 0.02.

Figure 3A **Figure 3B**

Fig. 3. Immunocytochemical detection of slow MyHC2 in muscle fibers treated with IP3R1 inhibitors. Non-stimulated (–Stim) and stimulated (+Stim) PM and MA muscle fibers were treated with 100uM 2-APB (A) and 10uM xestospongin D (B). Fast MyHC and slow MyHC2 were detected with monoclonal antibodies F59 and S58, respectively.

Figure 4A

Figure 4B

Fig. 4.

Cellular localization of NFAT. PM and MA muscle fibers, transfected with the expression construct encoding FLAG-tagged NFATc1, were immunostained with the F59 monoclonal antibody directed against fast MyHC and an anti-FLAG epitope antibody followed by FITC-conjugated and Texas Red-conjugated secondary antibodies, respectively. Nuclei were stained with DAPI. Images of fast MyHC and NFAT immunofluorescence were merged by addition of images using Photoshop CS 8.0.

Figure 5A

Figure 5C

Fig. 5.

Effects of electrical stimulation and IP3R1 inhibition on transcriptional regulators in PM and MA muscle fibers. Transcriptional sensors of NFAT (A), MEF2 (B), and SRE (C) activities were transfected into PM and MA muscle cells. Some of the cells were stimulated as before and/or treated with 100uM 2-APB to inhibit IP3R1 activity. Transcriptional sensor activities were measured by relative quantitation of firefly luciferase activities. Values are means $\pm$ SE, $n = 6$. * $P < 0.01$.

Figure 6A

Figure 6B

Fig. 6.

Effects of electrical stimulation and IP3R1 inhibition on slow MyHC2 promoter activity. The firefly luciferase gene transcriptionally controlled by either the wild type slow MyHC2 promoter in the DNA construct 2279SM2Luc (A) and or by the slow MyHC2 promoter harboring the NFAT binding site mutation (B) was transfected into PM and MA muscle cells. Some of the cells were stimulated as before and/or treated with 100uM 2-APB to inhibit IP3R1 activity. MyHC2 promoter activities were measured by relative quantitation of firefly luciferase activities. Values are means $\pm$ SE, n = 6. *P < 0.01.