Inhibition of ryanodine receptor 1 in fast skeletal muscle fibers induces a fast-to-slow muscle fiber type transition

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Summary

Skeletal muscle fiber type is regulated by innervation-induced cell signaling including calcium release mechanisms that lead to transcriptional activation of fiber type-specific genes. Avian fast pectoralis major (PM) and slow medial adductor (MA) muscles differentially control expression of the slow myosin heavy chain 2 (slow *MyHC2*) gene. We report here that slow *MyHC2* gene expression in fast PM muscle fibers is repressed by endogenous activity of the ryanodine receptor 1 (RyR1). Inhibition of RyR1 with ryanodine led to expression of the slow *MyHC2* gene in innervated PM muscle fibers in vitro. Administration of ryanodine to innervated PM muscle fibers also decreased protein kinase C (PKC) activity, the reduction of which is necessary for slow *MyHC2* gene expression in both PM and MA muscle fibers. Furthermore, RyR1 inhibition increased slow *MyHC2* promoter activity in innervated PM muscle fibers and enhanced transcriptional activities of nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2), as well as their interactions with their respective binding sites of the slow *MyHC2* promoter. These results indicate that RyR1 activity in innervated fast PM muscle fibers contributes to the cell type-specific repression of slow muscle specific genes.

Key words: Myosin heavy chain, Ryanodine, Avian, Skeletal muscle, Fiber type

Introduction

Vertebrate skeletal muscle consists of fast and slow contracting muscle fibers. In avian species, the pectoralis major muscle consists of muscle fibers expressing exclusively fast myosin heavy chain (MyHC) genes. The medial adductor muscle consists of fibers all of which express genes encoding both fast MyHCs and slow MyHC2 (Page et al., 1992). Expression of the slow *MyHC2* gene characterizes the definitive slow muscle fiber phenotype (Kennedy et al., 1986). Regulation of slow *MyHC2* gene expression and muscle fiber phenotype is mediated by combinatorial developmental mechanisms. During primary myogenesis, embryonic or primary myoblasts differentiate to form primary muscle fibers in the absence of functional innervation. A subset of these muscle fibers expresses the slow *MyHC2* gene as they first form, independently of innervation (Page et al., 1992). A subset of myotubes in vitro formed from embryonic myoblasts recapitulates this in vivo pattern of expression without innervation (Miller and Stockdale, 1986). Innervation-independent variation in muscle fiber phenotype based on individual myoblasts gave rise to the concept that intrinsic, cell lineage-dependent mechanisms yielded muscle fibers expressing contractile protein genes in fiber type specific patterns (Stockdale, 1992).

Similarly, a subset of avian secondary muscle fibers formed from fetal myoblasts expresses the slow *MyHC2* gene in vivo. However, this expression is partly dependent on functional innervation. Administration of curare during embryonic and fetal chick development reduced slow *MyHC2* gene expression in secondary muscle fibers of the medial adductor (Crow and Stockdale, 1986; DiMario and Funk, 1999). In addition, slow *MyHC2* gene expression in fetal muscle fibers is regulated in a fiber type dependent manner in vitro (DiMario and Stockdale, 1997). Muscle fibers formed from fetal medial adductor (MA) myoblasts in vitro expressed the slow *MyHC2* gene only when innervated. By contrast, muscle fibers formed from fetal pectoralis major (PM) myoblasts did not express the slow *MyHC2* gene, whether the fibers were innervated or not by the same pool of neurons.

The calcium-calmodulin-dependent protein phosphatase, calcineurin, has been proposed to be a mediator of a signaling cascade based on cell specific calcium transients leading to activation of muscle fiber type specific gene promoters. Its activity has been linked to dephosphorylation and activation of the transcription factor myocyte enhancer factor 2 (MEF2). MEF2 belongs to the family of MADS box transcription factors and binds to A/T-rich cis-elements in muscle-specific promoters. MEF2 binding sites are present in a variety of muscle-specific genes characteristic of both fast and slow muscle fiber types [e.g. MyHCIIa and troponin I-slow (TnIs) and myosin light chain 2 slow genes] (Dunn et al., 2001; Esser et al., 1999). MEF2 activity is dependent on its phosphorylation state (Wu et al., 2001). Dephosphorylation of MEF2 by calcineurin in response to nerve activity has been proposed as a mechanism for MEF2-mediated transcription of fiber type specific genes (Dunn et al., 2001).

Calcineurin also regulates activation of the nuclear factor of activated T cells (NFAT) transcription factor. Dephosphorylation of NFAT causes nuclear import and subsequent activation of NFAT-dependent promoters (Beals et al., 1997; Crabtree, 2001). The calcineurin-NFAT signaling pathway is potentially a mechanism by which cells decode complex calcium signaling. However, the complexity of calcium signaling has extended beyond the calcineurin-NFAT interactions, as it is now clear that multiple regulatory effects modulate the direct activation of NFAT by calcineurin. Some muscle-specific promoters such as the myoglobin and TnIs promoters are upregulated by constitutively active calcineurin (Chin et al., 1998). Therefore, on the basis of the activation of genes associated with the slow muscle fiber phenotype, it has been proposed that calcineurin-NFAT activation by slow muscle fiber calcium transients selectively regulates slow muscle fiber specific genes. This hypothesis was supported by in vivo studies in which inhibition of calcineurin by cyclosporine A reduced slow type I fiber composition in regenerating rat soleus muscle (Serrano et al., 2001). However, discordance in the unidirectional activation of slow muscle fiber specific genes by calcineurin activation of NFAT has also been reported. Calcineurin, but not NFATc3, increased slow MyHC gene expression in C_2C_{12} cells (Delling et al., 2000). Furthermore, fiber type specific calcium transients, calcineurin and NFAT also regulate fast muscle fiber specific genes. The fast *MyHCIIa* promoter was activated by increased calcium, NFAT and MEF2 (Allen and Leinwand, 2002). The avian slow *MyHC2* promoter in slow muscle fibers is activated by both NFAT and MEF2, and both are required for innervation-induced expression of the slow muscle fiber phenotype (Jiang et al., 2004). Furthermore, regulation of NFAT transcriptional activity was fiber-type dependent. Innervation of slow muscle fibers preferentially activated NFAT-dependent reporter genes relative to innervated fast muscle fibers. However, constitutively active NFAT in the presence of MEF2 activity was not sufficient to induce slow *MyHC2* promoter activity. In total, these studies indicated that NFAT and MEF2 participate in transcriptional regulation of muscle and fiber type specific genes and that fiber type specific mechanisms control activation of these factors.

Repression of slow *MyHC2* gene expression in innervated avian fast muscle fibers is mediated by a signal transduction cascade initiated by innervation itself. Release of acetylcholine from motor neurons at neuromuscular junctions is sufficient to activate vasodilation of muscle-associated arterioles via the muscarinic acetylcholine receptor (mAchR) as well as muscle fiber activity via the nicotinic acetylcholine receptor (nAchR) (Welsh and Segal, 1997). Inhibition of mAchR activity and associated signaling via Gαq in innervated fast muscle fibers is sufficient to induce slow *MyHC2* gene expression (Jordan et al., 2003). Similarly, slow *MyHC2* gene expression is induced in muscle fibers in which protein kinase C (PKC) activity is reduced (DiMario, 2001; DiMario and Funk, 1999). Conversely, slow *MyHC2* gene expression is repressed by increased PKC activity.

Excitation-contraction coupling in both fast and slow skeletal muscle fibers involves calcium release from intracellular stores. Intracellular calcium levels briefly rise to ~1 μM in fast fibers and to a more sustained increase of ~200 nM in slow fibers (Chin and Allen, 1996). The longer duration and lower amplitude calcium transient in slow muscle fibers is thought to activate calcineurin and NFAT (Timmerman et al., 1996; Dolmetsch et al., 1997). The initial release of calcium from the sarcoplasmic reticulum (SR) is mediated by the voltage-sensitive dihydropyridine receptor (DHPR) and by its physical interaction with the large sarcoplasmic reticulum (SR)-associated ryanodine receptor (RyR) – a tetrameric calcium release channel with individual polypeptide subunits of 565 kDa (Sutko and Airey, 1996). The RyR is also characterized by its high affinity for the plant alkaloid, ryanodine, which inhibits RyR-mediated calcium release. RyRs display single channel conductance resulting in localized transient increases in intracellular calcium in the form of calcium sparks (Nelson et al., 1995). Genes encoding three distinct isoforms of the RyR have been cloned from mammals and sequenced. The use of isoform-specific nucleotide probes and antibodies has shown a wide distribution of RyR isoforms in various tissues. RyR1 is the predominant RyR isoform in vertebrate skeletal muscle (Takeshima et al., 1989). RyR2 is abundant in cardiac muscle (Otsu et al., 1990), and RyR3 is present in brain tissue (Hakamata et al., 1992). In addition to the three mammalian RyR isoforms, a slow fiber type specific isoform, termed RyR1-slow, has been identified in fish skeletal muscle (Franck et al., 1998; Morrissette et al., 2000). However, to date no mammalian or avian fiber type specific variant isoform of RyR1 has been found.

The differential calcium transients of fast and slow muscle fibers, released partly by RyR1, potentially affect several signaling pathways. For instance, PKCα isoform activity, which is abundant in skeletal muscle, is dependent on calcium, and its activity is increased in fast versus slow muscle fibers (DiMario and Funk, 1999; Donnelly et al., 1994). Calcineurin is maximally activated by low amplitude, long duration calcium transients indicative of slow muscle fiber type calcium regulation (Dolmetsch et al., 1997). In addition, regulation of calcium release via RyR1 in skeletal muscle may be regulated by interactions between calcineurin and RyR1 via the RyR1 accessory protein, FKBP12 (Shin et al., 2002).

In the present study, the control of slow *MyHC2* gene expression and the slow muscle fiber phenotype by RyR activity in innervated fast and slow muscle fibers is examined. RyR activity was inhibited by ryanodine, and the effects on slow *MyHC2* gene expression and promoter activity were assessed. RyR activity-induced NFAT and MEF2 transcription factor activation were also assessed by the use of transcription factor-specific reporter genes and interactions with the slow *MyHC2* promoter.

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 (O'Neill and Stockdale, 1972) and plated at 4×10^5 cells per 35 mm collagen-coated dish in 10% horse serum (Hyclone), 5% chick embryo extract, in Ham's F-10 medium supplemented with 1.1 mM $CaCl_2$, and antibiotics (penicillin/streptomycin/Fungizone: Gibco). On day 3 of incubation, explants of ED5 chick embryo spinal cords containing motor neurons were added to the differentiated myotube cultures as previously described (DiMario and Stockdale, 1997). Cultures were maintained for a total of 7 days and medium was replaced every other day. For inhibition of

RyR1 activity, medium was supplemented with 100 μM ryanodine (Sigma) from days 4-7 of incubation.

Immunocytochemistry and western blots

Cells were immunostained on day 7 of incubation. All procedures were performed at room temperature. Cells were washed three times with phosphate buffered saline (PBS), pH 7.2, and then fixed. For immunodetection of fast MyHCs and slow MyHC2, cells were fixed with 100% ethanol for 5 minutes. For immunodetection of RyR1, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 10 minutes. Cells were washed three times with PBS and blocking solution consisting of 5% horse serum and 2% bovine serum albumin (BSA) in PBS was added to the cells for 1 hour. F59 and S58 monoclonal antibodies that specifically recognize fast MyHCs and slow MyHC2, respectively, were diluted 1:10 in blocking solution and added to the cells for 1 hour. The generation and specificities of these antibodies have been previously described (Crow and Stockdale, 1986). RyR1 antibody (Affinity Bioreagents) was diluted 1:100 in blocking solution and added to the cells for 1 hour. Cells were washed three times with PBS and incubated in fluorochrome-conjugated secondary antibodies diluted 1:100 in blocking solution. FITC-conjugated anti-rabbit IgG (Vector Labs) detected the RyR1 antibody. F59 and S58 antibodies were detected with Texas Red-conjugated anti-mouse IgG (Vector Labs) and FITC-conjugated anti-mouse IgA (Southern Biotech). Cells were washed three times with PBS, and a drop of 2.5% diazabicyclooctane in 90% glycerol was added before viewing by epifluorescence.

Protein for western blot analysis was extracted from ED13 whole muscle and muscle fiber cultures on day 7 of incubation using 0.5 ml 20 mM Tris, pH 7.5, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethyleneglycotetraacetic acid (EGTA), 0.5% Triton X-100, plus protease inhibitors (Boehringer Mannheim). Cells were homogenized in a glass dounce homogenizer, and the extracts were set on ice for 10 minutes. Protein extracts were clarified by centrifugation at 20,000 ***g*** for 5 minutes in an Eppendorf centrifuge. Protein concentrations were determined using a BCA protein assay reagent (Pierce). Protein was denatured at 95°C for 5 minutes in SDS-PAGE sample buffer, electrophoresed in a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated in blocking solution consisting of 2% nonfat dry milk, 0.05% Tween-20 in PBS for 2 hours. RyR1 and α-actin (Sigma) antibodies were diluted 1:2500 and 1:2000 in blocking solution, respectively, and incubated with the blots for 1 hour. Blots were washed three times with PBS and then incubated for 1 hour in horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotech) for RyR1 blots and HRP-conjugated anti-mouse IgM (Sigma) for α-actin blots diluted 1:500 and 1:1000, respectively. Blots were washed as before and developed by chemiluminescence (Pierce).

PKC activity assays

PKC activities were determined in muscle fiber cultures on day 7 of incubation. Cells were washed once with PBS and homogenized in cold 25 mM Tris, pH 7.2, 1 mM EDTA, 50 mM NaCl and protease inhibitors. Supernatants were clarified by centrifugation at 20,000 ***g*** for 10 minutes at 4°C in an Eppendorf centrifuge. Membrane pellets were resuspended in extraction buffer containing 0.5% Triton X-100 and set on ice for 30 minutes. The extracts were centrifuged as before and membrane protein fractions were collected. Protein concentrations were determined using a BCA protein assay reagent (Pierce). PKC activities were determined using a PKC assay kit (Upstart Biotech) according to the manufacturer's protocol.

DNA transfections

Myogenic cultures were transfected on day 2 of incubation with the full-length wild-type slow *MyHC2* promoter-luciferase reporter construct, 2279SM2Luc (3 μg/35 mm dish). This construct has been previously characterized (Jiang et al., 2004). NFAT and MEF2 sensor constructs containing multimerized NFAT and MEF2 binding sites, respectively, coupled to the luciferase gene (3 μg/35 mm dish) were kindly provided by E. Olson (University of Texas Southwestern Medical Center, Dallas, TX). The serum response element (SRE) sensor construct, SRELuc, was obtained from Stratagene. Transfection of pSVβGAL (1 μg/35 mm dish) served as a control of transfection efficiency. Constructs were transfected using Lipofectamine Plus (Invitrogen). Reporter gene assays were performed on day 7 of incubation. Luciferase activities from 2279SM2Luc and the NFAT, MEF2 and SRE sensors were normalized to β-galactosidase activities derived from pSVβGAL transfection.

Electromobility shift assays (EMSAs)

Nuclear extracts from PM and MA muscle fiber cultures were prepared as previously described (Parakati and DiMario, 2002). Protein content was determined by BCA protein assay (Pierce). Complementary oligonucleotides containing either the MEF2 or NFAT binding sites of the slow *MyHC2* promoter were commercially synthesized (Integrated DNA Technology). The MEF2 oligonucleotide consisted of the sequence, 5′ ACAGGAGTAAA-AATAACCAGGCTG 3′ and its complement. The NFAT oligonucleotide consisted of the sequence 5′ GAGGCAGAAA-GGAAAGCTCTCAGT 3′ and its complement. EMSA reactions were prepared as previously described (Parakati and DiMario, 2002). MEF2A and NFATc1 antibodies (4 μg) were added to the reaction mixtures and incubated overnight at 4°C. Protein-DNA complexes were resolved by 5% nondenaturing polyacrylamide gel electrophoresis with 1X Tris borate EDTA buffer. Gels were dried and exposed to x-ray film overnight or for 7 days (NFAT supershifts).

Statistics

Differences in mean values were assessed by Student's *t*-test with significant differences set at $P<0.05$.

Results

RyR1 activity regulates slow *MyHC2* gene expression

We have previously shown that expression of the slow *MyHC2* gene in avian MA muscle fibers in vitro was induced by innervation (DiMario and Stockdale, 1997). We have also shown that slow *MyHC2* promoter activity in innervated MA muscle fibers was dependent on the transcription factor, NFAT (Jiang et al., 2004). As NFAT activity is regulated by calcineurin, which has been proposed to differentially respond to cell-specific concentrations and transients of intracellular calcium, we hypothesized that the calcium release channel, RyR1, regulates slow *MyHC2* gene expression. RyR1 displays a biphasic response to the plant (Ryania speciosa) alkaloid, ryanodine. RyR1 channel conductance and kinetics are inhibited by high (100 μM) concentrations of ryanodine, whereas the frequency of channel opening is increased by low (10 nM-1 μM) ryanodine concentrations (Buck et al., 1992; Zimanyi et al., 1992). To determine whether RyR1 activity regulated slow *MyHC2* gene expression, innervated and noninnervated MA and PM muscle fibers were incubated in medium with and without 100 μM ryanodine. Cells were then immunostained with F59 and S58 monoclonal antibodies to detect fast MyHC(s) and slow MyHC2, respectively (Fig. 1). All muscle fibers expressed a fast MyHC(s) and

immunostained with F59. In agreement with previous findings, slow *MyHC2* gene expression was induced by innervation in MA muscle fibers and not in PM muscle fibers. Addition of 100 μM ryanodine to the culture medium for non-innervated MA and PM muscle fibers did not induce slow *MyHC2* gene expression. However, both innervated MA and PM muscle fibers incubated in medium containing ryanodine did immunostain with S58 and express the slow *MyHC2* gene. The presence of ryanodine was not necessary for slow *MyHC2* gene expression in innervated MA muscle fibers. Conversely, inclusion of ryanodine in the culture medium was necessary for slow *MyHC2* gene expression in innervated PM muscle fibers. Ryanodine at 50 μM and 75 μM did not elicit significant slow *MyHC2* gene expression as determined by immunostaining cells with S58 (data not shown). These results indicate that normal RyR1 activity in innervated fast PM muscle fibers represses slow *MyHC2* gene expression.

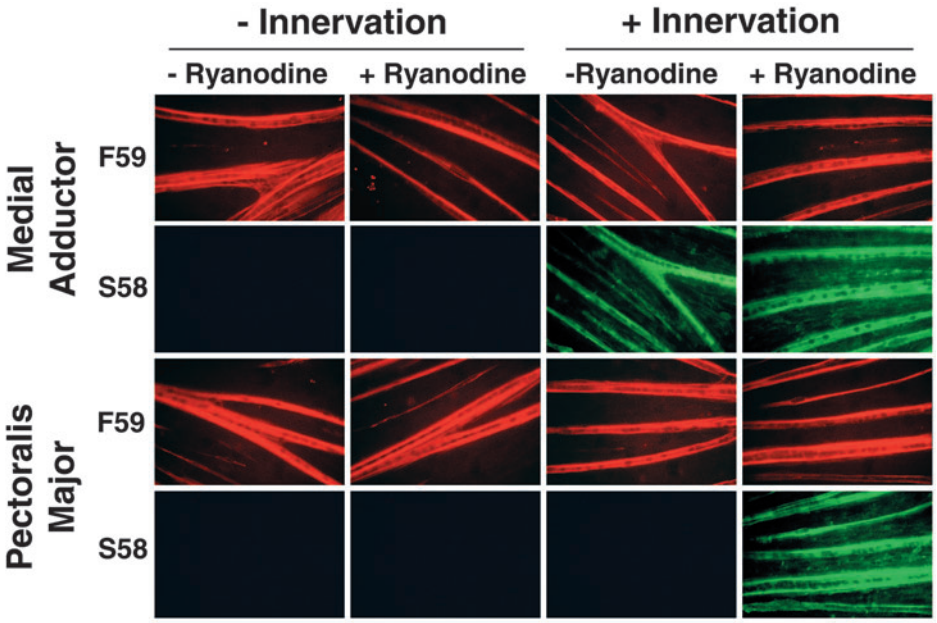

Fig. 1. Expression of slow MyHC2 in slow MA and fast PM muscle fibers. ED13 myoblasts from MA and PM muscles were incubated for 7 days. On the third day of incubation, some of the cultures received ED5 spinal cord explants to provide innervation. On day 4 of incubation, medium in some of the cultures was supplemented with 100 μM ryanodine. Muscle fibers were immunostained with F59 and S58 mAbs to detect fast MyHCs and slow MyHC2, respectively, followed by fluorochrome conjugated secondary antibodies. Slow MyHC2 was detected in innervated MA muscle fibers and in innervated PM muscle fibers cultured in medium containing 100 μM ryanodine.

Immunodetection of RyR1 in PM and MA muscle fiber cultures

Although three isoforms of RyR have been described, RyR1 is the predominant isoform expressed in skeletal muscle (McPherson and Campbell, 1997; Franzini-Armstrong and Jorgensen, 1994). RyR3 may be transiently expressed during development of some muscle fibers or exist in minor abundance (Flucher et al., 1999). To detect RyR1 in PM and MA muscle fibers in vitro, innervated and noninnervated cultures of each were immunostained with an RyR1-specific antibody (Fig. 2A). All muscle fibers contained RyR1. However, any obvious differences in the amount of RyR1 in these cultures were not observed. To more critically determine whether differences in RyR1 abundance existed in the different muscle fiber types, western blots of PM and MA muscle fibers were developed with the RyR1 antibody and quantitated (Fig. 2B,C). Western blots and RT-PCR analyses of muscle fiber cultures revealed expression of RyR1, but not RyR3 (data not shown). Extracts from ED13 PM in vivo contained significantly (P<0.03) more RyR1 than ED13 MA in vivo. Similarly, RyR1 was more abundant in noninnervated PM versus MA (P<0.03) and innervated PM versus MA (P<0.01). These results indicate that fast muscle fibers whether innervated or not contain significantly greater amounts of RyR1 relative to slow muscle fibers.

Ryanodine and innervation inhibit protein kinase C activity

We have previously shown that inhibition of PKC activity was sufficient to induce slow *MyHC2* gene expression in noninnervated MA muscle fibers and that PKC inhibition in conjunction with innervation induced slow *MyHC2* gene expression in fast PM muscle fibers (DiMario and Funk, 1999; DiMario, 2001). Furthermore, increased PKC activity repressed slow *MyHC2* gene expression. Therefore, we hypothesized that administration of ryanodine to PM and MA muscle fibers would lower PKC activity and that the ryanodine-induced pattern of slow *MyHC2* gene expression in PM muscle fibers correlated with reduced PKC activities. To address this hypothesis, PKC activities in innervated and noninnervated PM and MA muscle fiber cultures incubated in medium with or without 100 μM ryanodine were determined (Fig. 3). In PM muscle fibers, PKC activity was not significantly reduced by innervation or ryanodine alone. However, the combination of both innervation and ryanodine significantly reduced (P<0.05) PKC activity in these fibers. The combined effects of innervation and ryanodine had elicited slow *MyHC2* gene expression in fast PM muscle fibers (Fig. 1). In MA muscle fibers, innervation alone significantly reduced PKC activity relative to noninnervated control MA muscle fibers, and inhibition of RyR1 activity in innervated MA muscle fibers did not further reduce PKC activity. These results indicate that RyR1 activity in PM muscle fibers normally enhances PKC activity.

RyR1 activity regulates the slow *MyHC2* promoter

To determine the extent to which RyR1 activity regulates slow *MyHC2* gene expression, the slow *MyHC2* promoter,

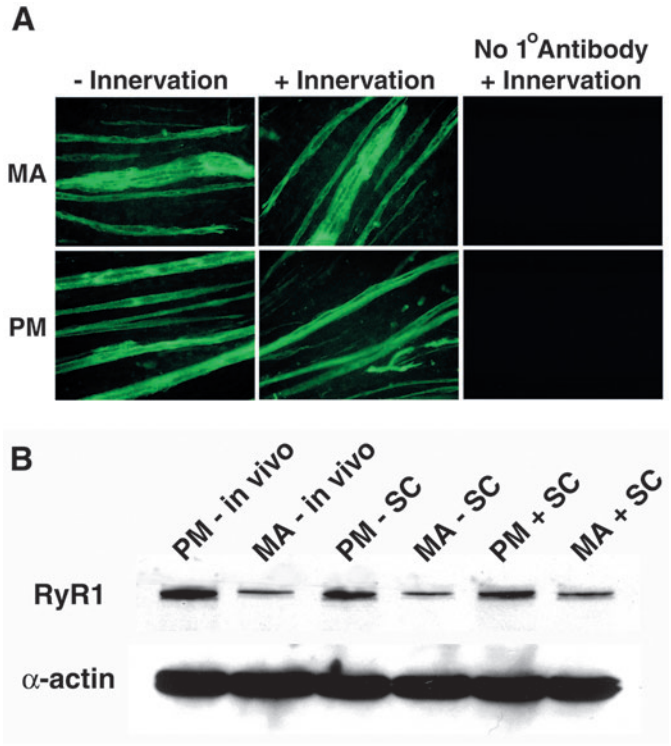

Fig. 2. Immunodetection of RyR1. (A) ED13 MA and PM muscle fibers with and without innervation were immunostained with an anti-RyR1 antibody and an FITC conjugated secondary antibody. Control for nonspecific immunostaining by the secondary antibody is included. (B) Protein extracts from ED13 PM and MA muscles in vivo as well as PM and MA muscle fibers cultured in the absence (–SC) and presence (+SC) of innervation were electrophoresed and blotted. RyR1 was detected using an anti-RyR1 antibody. α-Actin was used as a loading control for normalization of RyR1 abundance and was detected using an anti-sarcomeric α-actin antibody. (C) Western blots of RyR1 in extracts from ED13 PM and MA muscles in vivo as well as PM and MA muscle fibers cultured in the absence (–SC) and presence (+SC) of innervation were quantitated. PM muscle fibers contained significantly more RyR1 relative to MA muscle fibers (**P*<0.03; ***P*<0.01). Bars represent means±s.e.m.; *n*=5.

containing 1358 bp of DNA upstream from coding sequence, coupled to the luciferase reporter gene was transfected into PM and MA myogenic cultures. This slow *MyHC2* promoter sequence and its partial characterization have been previously reported (Jiang et al., 2004). Innervation was provided to half of the transfected PM and MA muscle fiber cultures by the addition of spinal cord explants, and cells were incubated in

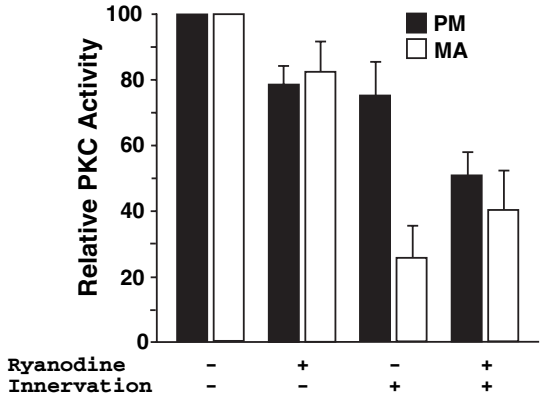

Fig. 3. PKC activity assays. Innervated and noninnervated PM and MA muscle fiber cultures incubated in control medium or medium containing 100 μM ryanodine were assayed for PKC activity. Results are expressed relative to PKC activities in noninnervated PM and MA muscle fibers incubated in control medium. PKC activities in PM muscle fibers in the presence of either innervation or 100 μM ryanodine were not significantly different. PM muscle fibers in the presence of both innervation and ryanodine had significantly less PKC activity ($P<0.05$). MA muscle fibers in the presence of innervation displayed significantly lower PKC activities ($P<0.05$). Bars represent means±s.e.m.; $n=5$.

control medium or medium containing 100 μM ryanodine. The resulting slow *MyHC2* promoter activities are presented in Fig. 4. Slow *MyHC2* promoter activity in MA muscle fibers was increased 2.5-fold by innervation in agreement with previous findings (Jiang et al., 2004). In PM muscle fiber cultures, addition of innervation or ryanodine alone did not significantly increase slow *MyHC2* promoter activity. However, addition of both innervation and 100 μM ryanodine caused a significant ($P<0.02$) 2.3-fold increase in slow *MyHC2* promoter activity. These results are in agreement with the immunofluorescent detection of slow MyHC2 in PM muscle fibers cultured only in the presence of both innervation and ryanodine (Fig. 1).

RyR1 activity regulates NFAT and MEF2 activities

Transcription of the slow *MyHC2* gene is dependent on NFAT and MEF2 transcription factors (Jiang et al., 2004). In addition, we have previously shown that NFAT transcriptional activity in MA muscle fibers is regulated by innervation. Therefore, we hypothesized that the regulation of slow *MyHC2* gene expression by RyR1 in PM muscle fibers was also mediated by regulation of NFAT and MEF2 transcription factor activities. To determine the effects of ryanodine treatment on NFAT and MEF2 activities, NFATLuc and MEF2Luc sensor constructs were individually transfected into PM myogenic cultures. Half of the muscle fiber cultures received spinal cord explants with control medium or with medium containing 100 μM ryanodine. NFAT-mediated transcriptional activity was not significantly affected in PM muscle fibers by innervation or ryanodine alone compared with noninnervated muscle fibers in control medium (Fig. 5). However, the combination of innervation and ryanodine caused a significant ($P<0.05$) increase of 2.6-fold in NFAT-mediated transcription. Similar results were obtained with the MEF2Luc sensor (Fig. 5). Innervation or ryanodine treatment alone did not significantly affect MEF2-mediated

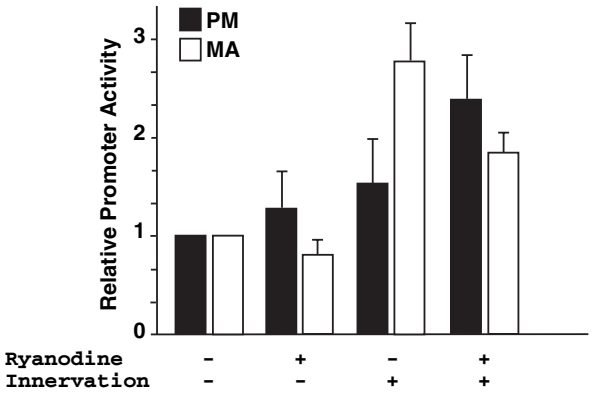

Fig. 4. Effects of ryanodine and innervation on slow *MyHC2* promoter activity. PM and MA muscle fibers transfected with the slow *MyHC2* promoter-luciferase construct, 2279SM2Luc, were innervated by spinal cord explants and cultured in medium containing 100 μM ryanodine. Slow *MyHC2* promoter activities were normalized by cotransfection of pSVβGAL and expressed relative to promoter activities in noninnervated PM and MA muscle fibers incubated in control medium. Innervation significantly increased promoter activity in MA muscle fibers (P<0.05). Combinatorial effects of innervation and ryanodine significantly increased promoter activity in PM muscle fibers (P<0.05). Bars represent means±s.e.m.; n=6.

transactivation of the MEF2Luc sensor. The combinatorial effects of innervation and RyR1 inhibition caused a twofold increase (P<0.02) in MEF2 activity relative to noninnervated muscle fibers in control medium. In addition, incubation of innervated muscle fibers in medium containing ryanodine caused a significant (P<0.05) increase in MEF2 activity relative to innervated muscle fibers in control medium. As a control to determine whether the increased transcriptional activity was specific to NFAT and MEF2 transcription factors versus a generalized increase in transcription factor function, SREluc containing the serum response element (SRE) binding site for serum response factor was also transfected into the myogenic cells. Transcriptional activity via the SRE of the SRELuc sensor did not increase under these experimental conditions (Fig. 5, lower panel). These results indicate that inhibition of RyR1 enhances NFAT and MEF2-mediated transcription.

RyR1 inhibition increases NFAT and MEF2 binding to their respective binding sites in the slow *MyHC2* promoter

NFAT and MEF2 transcription factor activities in skeletal muscle are dependent on calcium signaling events and innervation (Jiang et al., 2004). To determine whether NFAT and MEF2 occupancy of their respective binding sites in the slow *MyHC2* promoter was regulated by RyR1 activity, electromobility shift and supershift assays were performed using nuclear extracts from PM and MA muscle fibers cultured in the absence and presence of innervation as well as 100 μM ryanodine. Nuclear extracts of innervated PM muscle fibers cultured in medium containing 100 μM ryanodine formed a protein-DNA complex with the slow MyHC2 NFAT binding site (Fig. 6A). Nuclear extracts from PM muscle fibers without

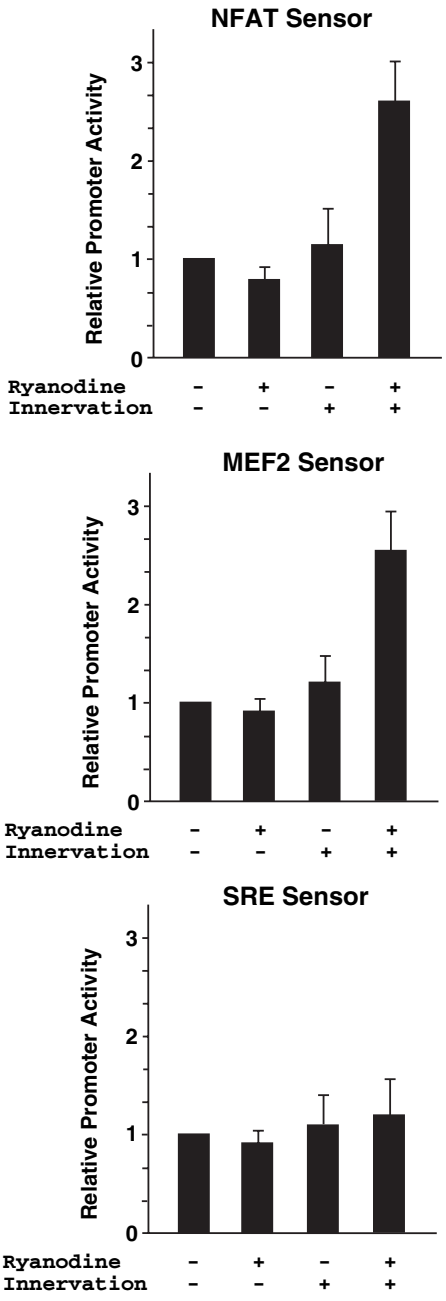

Fig. 5. Effects of RyR1 inhibition on NFAT and MEF2-mediated transcription. PM muscle fibers transfected with either NFATLuc or MEF2Luc sensor constructs were co-cultured with spinal cord explants for innervation with and without 100 μM ryanodine in the culture medium. Data are expressed relative to sensor activities in noninnervated PM muscle fibers incubated in control medium. Innervation or ryanodine alone did not significantly increase NFAT or MEF2-mediated transcription, whereas both innervation and ryanodine significantly increased both NFAT and MEF2 transcriptional activity (P<0.05). SRE-mediated transcriptional activity did not significantly increase with ryanodine treatment or innervation, or both. Bars represent means±s.e.m.; n=6.

innervation or ryanodine did not form this complex. In addition, innervated PM muscle fibers cultured in control medium and noninnervated PM muscle fibers cultured in

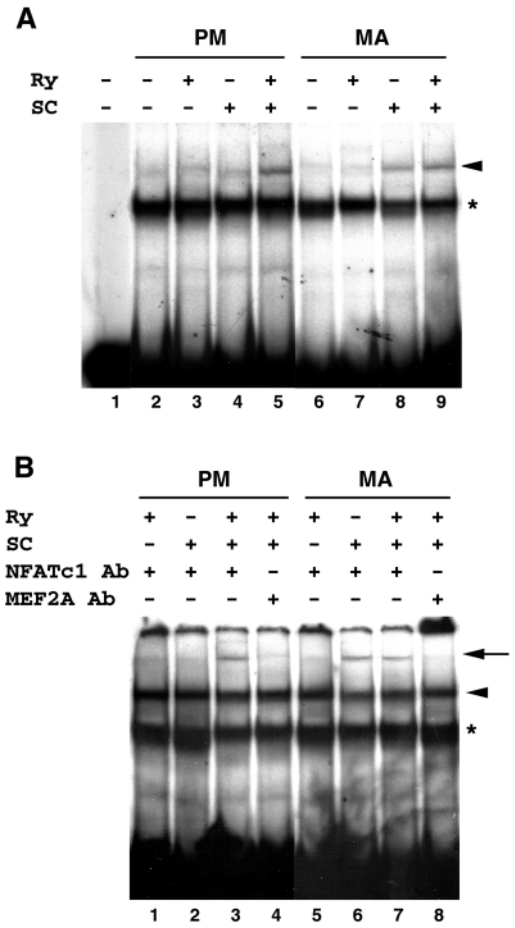

Fig. 6. Electromobility shift and supershift assays of the slow MyHC2 NFAT binding site. (A) Nuclear extracts from innervated (SC) and noninnervated PM (lanes 2-5) and MA (lanes 6-9) muscle fibers incubated in control medium or medium containing 100 μM ryanodine (Ry) were incubated with the NFAT binding site oligonucleotide probe. Protein-DNA complexes were resolved in 5% nondenaturing polyacrylamide gel. A specific protein-DNA complex (arrowhead) formed from extracts of innervated PM muscle fibers incubated in medium containing ryanodine (lane 5) and from extracts of innervated MA muscle fibers (lanes 8 and 9). Lane 1 contained no nuclear extract. A faster migrating protein-DNA complex (asterisk) of unknown composition was present in each lane. (B) Inclusion of an anti-NFATc1 antibody in the binding reaction resulted in a supershifted protein-DNA complex (arrow). An anti-MEF2a antibody did not yield a supershifted complex.

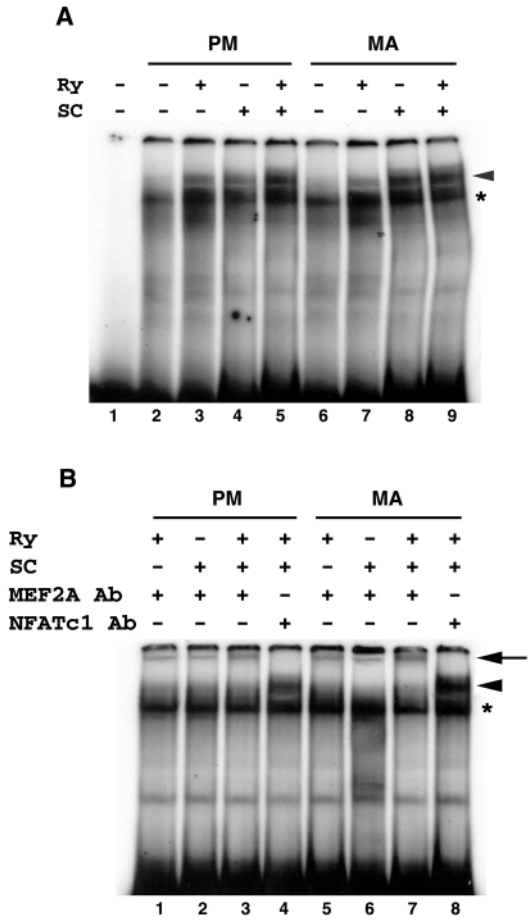

Fig. 7. Electromobility shift and supershift assays of the slow MyHC2 MEF2 binding site. (A) Nuclear extracts from innervated (SC) and noninnervated PM and MA muscle fibers were incubated with the MEF2 binding site oligonucleotide. Lane 1 contained no nuclear extract. Lanes 2-5 contained PM nuclear extract, and lanes 6-9 contained MA nuclear extracts. A protein-DNA complex (arrowhead) was identified in lanes 3-5 and 7-9 as formed by nuclear extracts from PM and MA muscle fibers either innervated or incubated in medium containing ryanodine. A faster migrating protein-DNA complex (asterisk) of unknown composition was present in each lane. (B) Inclusion of anti-MEF2A antibody in the binding reaction caused the formation of a supershifted complex (arrow). Inclusion of a NFATc1 antibody did not decrease the mobility of the MEF2A-containing complex.

medium with ryanodine did not form this protein-DNA complex. Nuclear extracts from innervated MA muscle fibers formed a similar protein-DNA complex with and without ryanodine. However, no protein-DNA complex was detected from nuclear extracts without innervation. The differentially regulated protein-DNA complex formed by PM and MA nuclear extracts was supershifted by inclusion of an NFATc1 antibody (Fig. 6B). These results indicate that RyR1 activity in innervated PM muscle fibers inhibits NFATc1 occupancy of the slow *MyHC2* promoter which is required for full promoter activation.

Electromobility shift assays of the same nuclear extracts with the slow MyHC2 MEF2 binding site oligonucleotide yielded similar, but not identical, results (Fig. 7A). Addition of ryanodine to the culture medium caused the formation of a specific protein-DNA complex. Similarly, innervation of PM muscle fibers caused the formation of a shifted complex. Formation of this complex was enhanced by the addition of ryanodine to innervated PM muscle fibers. A shifted complex formed using extracts from noninnervated MA muscle fibers cultured in medium containing ryanodine. Formation of this complex was enhanced using extracts from innervated MA muscle fibers in control medium, but was not obviously further enhanced by addition of ryanodine to innervated MA muscle fibers. The specific protein-DNA complex formed by PM and MA nuclear extracts was supershifted by inclusion of a MEF2A antibody (Fig. 7B). These results indicate that RyR1 activity

inhibits MEF2 occupancy of the slow *MyHC2* promoter which is required for full promoter activity in PM muscle fibers. The combinatorial effects of innervation and RyR1 inhibition in PM muscle fibers resulted in maximal MEF2 binding to the slow *MyHC2* promoter, and it was these conditions that resulted in slow *MyHC2* gene expression (Fig. 1).

Discussion

The RyR in skeletal muscle fibers releases Ca^{2+} from the SR as a transient Ca^{2+} spark. The release of Ca^{2+} is a necessary component of excitation-contraction coupling. In addition, intracellular Ca^{2+} regulates activities of signaling molecules such as PKC and transcription factors such as NFAT and MEF2 (Crabtree, 2001; Wu et al., 2001; Dunn et al., 2001). In fast contracting PM muscle fibers, intracellular Ca^{2+} levels rise to approximately 1 μM and in slow contracting MA muscle fibers the peak of intracellular free calcium is approximately 200 nM. Although lower in amplitude, the free Ca^{2+} transient in slow muscle fibers is typically of longer duration because of decreased re-uptake by SERCA and reduced cytoplasmic Ca^{2+}-binding proteins (Celio and Heizmann, 1983). The lower amplitude, longer duration Ca^{2+} transient observed in slow muscle fibers has been hypothesized to facilitate activation of the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, which dephosphorylates and activates NFAT and MEF2 (Crabtree, 2001; Wu et al., 2001).

Secondary muscle fibers of chicken skeletal muscle express either fast MyHC genes exclusively or both fast MyHC genes and slow *MyHC2*. Therefore, expression of the slow *MyHC2* gene is a significant distinguishing feature of diverse fiber types in chicken muscle. Partial characterization of the transcriptional regulatory mechanism of the slow *MyHC2* gene has been reported (Jiang et al., 2004). Slow *MyHC2* promoter activity is dependent on innervation and the transcription factors NFAT and MEF2. Furthermore, regulation of slow *MyHC2* gene expression was restricted in a fiber type specific manner. Innervation was sufficient to induce NFAT activation, slow *MyHC2* promoter activation and expression of the endogenous slow *MyHC2* gene in slow MA muscle fibers. However, innervation of fast PM muscle fibers did not induce NFAT transcriptional activity or slow *MyHC2* promoter activity.

On the basis of the role of RyR as a regulator of innervation-induced Ca^{2+} release and thereby intracellular Ca^{2+} concentration, and on the basis of the knowledge that slow *MyHC2* promoter activity is dependent on innervation, NFAT and MEF2, we hypothesized that disruption of RyR activity in skeletal muscle cells would affect slow *MyHC2* gene expression. More specifically, we hypothesized that reduction of RyR activity by ryanodine would induce slow *MyHC2* gene expression in innervated fast PM muscle fibers, mimicking the pattern of gene expression displayed by innervated slow MA muscle fibers with normal RyR activity. As a corollary hypothesis, RyR activity normally inhibits slow *MyHC2* gene expression in fast PM muscle fibers.

To address these hypotheses, the well-defined RyR1 inhibitor, ryanodine, was added to cultures of innervated and noninnervated PM and MA muscle fibers. As previously observed, innervated MA muscle fibers expressed the slow *MyHC2* gene, whereas innervated PM muscle fibers in control medium did not. However, inclusion of 100 μM ryanodine in the medium of innervated PM muscle fibers induced slow *MyHC2* gene expression. These results indicate that fiber type identity based on MyHC gene expression is dependent on innervation and regulated Ca^{2+} release via the RyR1 after muscle fiber depolarization.

Multiple signaling molecules regulate slow *MyHC2* gene expression in MA and PM muscle fibers. Many of these signaling molecules and transcription factors are themselves regulated by intracellular Ca^{2+}. Slow *MyHC2* gene expression is repressed in PM muscle fibers by Gαq signaling leading to increased PKC activity (Jordan et al., 2003). In noninnervated MA muscle fibers, slow *MyHC2* gene expression is induced by a reduction of PKC activity, and slow *MyHC2* gene expression is repressed in innervated MA muscle fibers by increased PKC activity (DiMario, 2001; DiMario and Funk, 1999). PKCα, a predominant PKC isoform in skeletal muscle, is activated by Ca^{2+}. Therefore, we hypothesized that PKC activity is dependent on RyR1 activity and that inhibition of RyR1 activity would decrease PKC activity. Addition of ryanodine to noninnervated PM and MA muscle fibers did not significantly reduce PKC activity. This was probably due to the inability of ryanodine to inhibit RyR activity in muscle fibers in which RyR was not activated by innervation. Addition to innervated PM muscle fibers did significantly reduce PKC activity. The data suggest that Ca^{2+} release via PM RyR1, which is two to three times more abundant than MA RyR1, results in a high amplitude Ca^{2+} spike, providing a positive regulator of PKC activity. This is further suggested by previous reports of increased PKC activity in fast versus slow avian and mammalian muscle fibers (DiMario and Funk, 1999; Donnelly et al., 1994).

Regulated Ca^{2+} release has also been proposed as a mechanism by which the activities of transcription factors NFAT and MEF2 are controlled. The slow *MyHC2* promoter is dependent on NFAT and MEF2 transactivation in MA muscle fibers (Jiang et al., 2004). To determine whether inhibition of RyR1 by ryanodine affected slow *MyHC2* promoter activity, innervated and noninnervated PM and MA muscle fibers transfected with the slow *MyHC2* promoter-luciferase construct were incubated in control medium and medium with 100 μM ryanodine. As shown previously (Jiang et al., 2004), slow *MyHC2* promoter activity significantly increased in MA muscle fibers because of innervation. In PM muscle fibers, innervation or ryanodine treatment alone did not significantly increase promoter activity in fast PM muscle fibers. However, the combinatorial effects of innervation and ryanodine treatment significantly increased slow *MyHC2* promoter activity in PM muscle fibers. This increase in promoter activity was reflected by significant increases in NFAT- and MEF2-mediated transcriptional activity. Additionally, activation of the slow *MyHC2* promoter by innervation and ryanodine was reflected by occupancy of the NFAT and MEF2 binding sites within the promoter by NFATc1 and MEF2A.

These results suggest that RyR1 activity in innervated PM and MA muscle fibers differentially regulates slow *MyHC2* gene expression. In MA muscle fibers, slow *MyHC2* gene expression is positively regulated by innervation and the subsequent release of Ca^{2+} via RyR1 activity. However, in fast PM muscle fibers, innervation represses slow *MyHC2* gene expression via RyR1 activity. It is likely that the fiber type specific activity of RyR1 controls Ca^{2+} release to establish fiber type specific conditions that either activate (MA muscle fibers)

or repress (PM muscle fibers) slow *MyHC2* gene expression. Further evidence of this regulation was detected by decreased slow *MyHC2* promoter activity in innervated MA muscle fibers in the presence of ryanodine. These results suggest the testable hypothesis that defined ranges of intracellular Ca^{2+} regulate downstream effector molecules such as PKC, NFAT and MEF2. Increased Ca^{2+} amplitudes via increased RyR1 activity, characteristic of fast muscle fibers, appear to activate signaling components (e.g. PKC) that repress slow *MyHC2* gene expression. Conversely, the lower amplitude, longer duration Ca^{2+} transient characteristic of slow muscle fibers may activate slow MyHC2 gene transcription.

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