Regulation of skeletal muscle fiber type and slow myosin heavy chain 2 gene expression by inositol trisphosphate receptor 1

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Summary
Innervation-dependent signaling cascades that control activation of downstream transcription factors regulate expression of skeletal muscle fiber type-specific genes. Many of the innervation-regulated signaling cascades in skeletal muscle are dependent on intracellular calcium and the mechanisms by which calcium is released from the sarcoplasmic reticulum (SR). We report that the inositol trisphosphate receptor 1 (IP3R1) activity for calcium release from the SR as a slow wave, was more abundant in fast contracting compared to slow contracting avian muscle fibers. Furthermore, inhibition of IP3R1 activity by 2-aminoethoxydiphenylborate (2-APB) and xestospongin D induced a fiber type transition and expression of the slow myosin heavy chain 2 (slow MyHC2) gene in innervated fast muscle fibers. Activation of the slow MyHC2 promoter by IP3R1 inhibition was accompanied by a reduction in protein kinase C activity. In addition, inhibition of IP3R1 activity resulted in a reduction of nuclear factor of activated T cells (NFAT)-dependent transcription and nuclear localization, indicating that IP3R1 activity regulated NFAT transcription factor activity in skeletal muscle fibers. Myocyte enhancer factor 2 (MEF2)-dependent transcriptional activity was increased by innervation, but unaffected by IP3R1 activity. The results indicate that IP3R1 activity regulates muscle fiber type-specific gene expression in innervated muscle fibers.

Key words: Myosin heavy chain, IP3R1, Calcium, Avian, Fiber type, NFAT, MEF2

Introduction
Vertebrate skeletal muscle consists of distinct muscle fiber cell phenotypes defined by expression of fiber type-specific subsets of genes encoding contractile and non-contractile proteins. Avian skeletal muscle consists of fast and slow contracting muscle fibers. Nearly all avian muscle fibers express a fast myosin heavy chain (MyHC) gene(s). Phenotypic variation in fiber types is largely defined by expression of slow MyHC genes, particularly the slow MyHC2 gene, in some of the muscle fibers (Page et al., 1992). Examples of muscles with these distinct phenotypes include the exclusively fast fiber pectoralis major (PM) and the medial adductor (MA), which uniformly contains fibers that express both fast MyHC genes and the slow MyHC2 gene.

In fetal secondary muscle fibers, slow MyHC2 gene expression is regulated by innervation and intrinsic muscle fiber type-specific characteristics. Muscles denervated in vivo by curare contained an increased percentage of fast compared to slow muscle fibers (Crow and Stockdale, 1986). This was most evident in the curare-treated fetal MA in which secondary muscle fibers did not contain detectable levels of slow MyHC2 (DiMario and Funk, 1999). Normal innervation-dependent regulation of slow MyHC2 gene expression was observed in vitro by innervation of MA muscle fibers and subsequent slow MyHC2 gene expression (DiMario and Stockdale, 1997). Fast PM muscle fibers in vitro were resistant to innervation-induced slow MyHC2 gene expression, suggesting a muscle fiber type-specific mechanism of slow MyHC2 gene repression in these fibers.

Fiber type-specific cell signaling cascades regulate slow MyHC2 gene expression and muscle fiber type. Cell signaling initiated by muscarinic acetylcholine receptor activity and transduced via Gαq to phospholipase C activity repressed slow MyHC2 gene expression in innervated fast PM muscle fibers. Inhibition of the muscarinic acetylcholine receptor by atropine resulted in slow MyHC2 gene expression in innervated PM muscle fibers (Jordan et al., 2003). Similarly, overexpression of wild-type PKCα repressed slow MyHC2 expression, whereas overexpression of a dominant-negative PKCα mutant induced slow MyHC2 gene expression in innervated PM muscle fibers (DiMario, 2001).

Muscle fiber depolarization elicits release of calcium from intracellular stores. Initial calcium release from the SR is regulated by the ryanodine receptor (RyR). Calcium release via the RyR calcium channel occurs as a short duration, high amplitude spark. This mode of increase of free intracellular calcium has been postulated to be inhibitory to activation of the calcium responsive phosphatase calcineurin and resulting downstream dephosphorylation and activation of the transcription factor, nuclear factor of activated T cells (NFAT) (Chin et al., 1998). In support of this model, inhibition of RyR...
activity in innervated fast PM muscle fibers increased NFAT transcriptional activity and slow MyHC2 promoter activity resulting in a fiber type transition (Jordan et al., 2004).

RyRs belong to a class of large tetrameric calcium release channels, which also includes inositol trisphosphate receptors (IP3Rs). Avian and mammalian species express three isoforms of IP3R, and their expression is widespread throughout numerous cell types. IP3R1 is present in skeletal muscle and the cerebellum, and type IP3R2 is abundant in cardiac tissue. IP3Rs contain more than 2700 amino acid residues that are highly conserved across species. The binding domain for inositol 1,4,5-trisphosphate (IP3) exists at the N-terminus. A large regulatory domain contains binding sites for ATP and calcium as well as phosphorylation sites and domains for interactions with regulatory proteins such as FKBP12. The C-terminus contains the ion channel pore. IP3Rs generally reside in the endoplasmic reticulum, although IP3Rs have been localized to the plasma membrane and nuclei (Kuno and Gardner, 1987; Nicotera et al., 1990). In skeletal muscle, IP3R1 has been localized to the SR and postsynaptic components of the neuromuscular junction (Powell et al., 2001; Powell et al., 2003). Activation of phospholipase C signaling pathways results in generation of the second messenger, IP3, which binds to its receptor on the SR. In many cell types IP3 agonist stimulation of the IP3R results in repetitive transient increases in intracellular calcium. Release of calcium in these cells is propagated as waves (Rooney et al., 1990; Lechleiter et al., 1991). In skeletal muscle, ligand binding results in calcium release from the SR as a slow wave relative to the RyR-mediated calcium spark. This slow wave of calcium release can be blocked by the IP3R inhibitors 2-aminoethoxydiphenylborate (2-APB) and xestospongin D and by the phospholipase C inhibitor U-73122 (Powell et al., 2001).

Slow MyHC2 gene expression in slow MA muscle fibers is dependent on both MEF2 and NFAT transcription factor activity. Mutation of MEF2 and NFAT binding sites within the slow MyHC2 promoter repressed innervation-induced slow MyHC2 promoter activity (Jiang et al., 2004). Based on the findings that slow MyHC2 gene expression is dependent on MEF2 and NFAT, both of which have been reported to be dependent on calcium-based cell signaling, we hypothesized that IP3R activity in innervated muscle fibers in vitro regulates slow MyHC2 gene expression and muscle fiber type. We report here that inhibition of IP3R activity in innervated PM muscle fibers elicits expression of the slow MyHC2 gene and a fast-to-slow muscle fiber type conversion.

Materials and Methods

Cell culture

Myoblasts were isolated from embryonic day 13 (E13) PM and MA muscles as previously described (O’Neill and Stockdale, 1972) and plated at 4 x 10⁶ cells per 35 mm collagen coated dish in 10% horse serum (Hyclone), 5% chick embryo extract, in Ham’s F-10 base medium supplemented with 1.32 mM CaCl₂ and antibiotics (penicillin, streptomycin, Fungizone; Gibco). Spinal cord explants containing motor neurons from E5 chick embryos were added to some of the cultures on day 3 of incubation as previously described (DiMario and Stockdale, 1997). On day 4 of incubation, medium for some of the cultures was supplemented with 100 µM 2-aminoethoxydiphenylborate (2-APB) (Sigma) or 10 µM xestospongin D (Calbiochem). Cultures were maintained for a total of 7 days with medium replaced every other day.

Immunocytochemistry and western blots

Cells in culture were immunostained on day 7 of incubation. Cells were washed three times with phosphate-buffered saline (PBS) and then fixed. For immunodetection of fast MyHC and slow MyHC2, cells were fixed with 100% ethanol for 5 minutes at room temperature. For immunodetection of IP3R1, cells were fixed with 3.7% formaldehyde followed by permeabilization with 0.1% Triton X-100 for 10 minutes. Cells were washed with PBS three times and blocking solution consisting of 5% horse serum and 2% bovine serum albumin (BSA) in PBS was added to the cells for 1 hour at room temperature. Monoclonal antibodies F59 and S58 specifically recognize fast MyHCs and slow MyHC2, respectively. These antibodies have been previously characterized (Crow and Stockdale, 1986). Both F59 (IgG) and S58 (IgA) monoclonal supernatants were diluted 1:10 in blocking solution and added to the cells for 1 hour at room temperature. For immunodetection of IP3R1, a rabbit polyclonal antibody was generated (Cambridge Research Biochemicals) against the chicken IP3R1 peptide, DAGELDNPKKFRDC, located near the N-terminus of the protein. This antibody was diluted 1:1000 in blocking solution and added to the cells for 1 hour at room temperature. After primary antibody incubation, cells were washed with PBS as before. F59, S58 and the IP3R1 antibody were detected by fluorescein-conjugated anti-mouse IgG (Vector Labs), Texas Red-conjugated anti-mouse IgA (Southern Biotech) and fluorescein-conjugated anti-rabbit IgG (Vector Labs), respectively, all diluted 1:100 in blocking solution. Secondary antibodies were incubated with the cells for 1 hour at room temperature followed by three washes with PBS. Clusters of acetylcholine receptors in innervated muscle fibers were visualized by addition of 100 nM rhodamine-conjugated α-bungarotoxin (Molecular Probes) for 1 hour at 37°C prior to fixation. A drop of 2.5% diazabicyclooctane in 90% glycerol and a coverslip were applied before viewing by fluorescence microscopy.

Total protein was extracted from PM and MA muscle fiber cultures on day 7 of incubation for western blot analyses of IP3R1 and α-actin. Cells were washed with cold PBS and scraped in homogenization buffer comprising 20 mM Tris-HCl, pH 7.5, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethyleneglycoltetraacetic acid (EGTA), 0.5% Triton X-100 and protease inhibitors (Boehringer Mannheim). Cells were homogenized in a glass dounce homogenizer, and the extracts were placed on ice for 10 minutes. Supernatants were collected after centrifugation at 20,000 g in an Eppendorf centrifuge at 4°C. Protein concentrations were determined by BCA protein assay (Pierce). Protein was denatured at 95°C for 5 minutes, electrophoresed in a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated in blocking solution consisting of 2% non-fat dry milk, 0.05% Tween-20 in PBS for 2 hours. IP3R1 and α-actin (Sigma) antibodies were diluted 1:1000 and 1:2000, respectively, in blocking solution and incubated with the blots for 1 hour at room temperature. Blots were washed three times in PBS/Tween for 5 minutes each. Blots were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) for IP3R1 blots and HRP-conjugated anti-mouse IgM (Sigma) for actin blots diluted 1:500 and 1:1000, respectively, for 1 hour at room temperature. Blots were washed as before and developed by chemiluminescence (Pierce). Protein (200 µg) was electrophoresed and blotted as above. NFATc1 and MEF2A antibodies (Santa Cruz Biotechnology) were diluted 1:500. Immunoblots were processed as above.
PKC activity assays
PKC activities were determined in membrane fractions of muscle fiber cultures on day 7 of incubation. Cells were washed with PBS and homogenized in cold 25 mM Tris-HCl, pH 7.2, 1 mM EDTA, 50 mM NaCl, and protease inhibitors. Pellets were collected by centrifugation at 20,000 g in an Eppendorf centrifuge for 10 minutes at 4°C. Membrane pellets were resuspended in extraction buffer containing 0.5% Triton X-100 and placed on ice for 30 minutes. The extracts were centrifuged as before, and the protein supernatants were collected. Protein concentrations were determined using a BCA protein assay (Pierce). PKC activities were determined using a PKC assay kit according to the manufacturer’s protocol (Upstate Biotechnology).

DNA transfections
Myogenic cultures were transfected (3 μg/35 mm dish) on day 2 of incubation with the full-length slow MyHC2 promoter-reporter gene construct, 2279SM2Luc (Jiang et al., 2004). NFAT and MEF2 sensor constructs containing multiple NFAT and MEF2 binding sites driving transcription of the luciferase reporter gene (kindly provided by E. Olson, UT Southwestern, Dallas, TX) were also transfected into myogenic cultures on day 2 of incubation. To control for variation in transfection efficiencies, pSVβGAL (1 μg/35 mm dish) was also transfected. DNAs were transfected using Lipofectamine Plus (Invitrogen). Luciferase and β-galactosidase activities (Sambrook et al., 1989) were determined on day 7 of incubation.

Results
Immunodetection of IP3R1 in PM and MA muscle fibers and extracts
PM and MA muscle fibers in vitro were derived from myoblasts isolated from ED13 chick PM and MA muscles. Some of the muscle fibers were innervated by spinal cord explants isolated from ED5 chick embryos (DiMario and Stockdale, 1997). Muscle fibers were immunostained with an IP3R1-specific antibody (Fig. 1). Both PM and MA muscle fibers contained detectable levels of IP3R1. Innervation of muscle fibers, as detected by visualization of acetylcholine receptor clusters with rhodamine-conjugated α-bungarotoxin, did not result in an observable difference in IP3R1 localization compared to that in non-innervated muscle fibers.

To determine whether innervated and non-innervated PM and MA muscle fibers expressed different amounts of IP3R1, western blots were performed. Whole cell extracts of PM and MA muscle fibers were electrophoresed, blotted and incubated with the IP3R1 antibody (Fig. 2A). Immunodetection of α-actin was used to control for protein loading. A protein band of approximately 300 kDa was detected in each lane containing muscle fiber cell extract. The relative mobility of the protein corresponds to previously published results (Supattapone et al., 1988). The IP3R1 bands from innervated and non-innervated PM and MA muscle fibers were quantified and compared (Fig. 2B). Non-innervated PM muscle fiber extracts contained approximately sixfold greater amounts of IP3R1 than non-innervated MA muscle fiber extracts. Similarly, innervated PM muscle fiber extracts contained approximately 3.5-fold greater IP3R1 than innervated MA muscle fiber extracts. Innervation of both PM and MA muscle fibers significantly (P<0.05) increased IP3R1 content by two- and 3.3-fold, respectively.

Inhibition of IP3R induces slow MyHC2 gene expression in innervated fast PM muscle fibers
IP3Rs regulate intracellular calcium release in many cell types, and as intracellular calcium concentrations regulate downstream signaling molecules, some of which are implicated in the regulation of muscle fiber type, we hypothesized that IP3R1 activity in skeletal muscle fibers contributed to muscle fiber phenotype. To determine whether IP3R1 activity regulated muscle fiber phenotype, innervated and non-innervated PM and MA muscle fibers were cultured in control medium or medium containing either the

Fig. 1. Immunodetection of IP3R1 in fast and slow muscle fibers. Embryonic day 13 (E13) pectoralis major and medial adductor myoblasts were isolated, cultured, and allowed to differentiate into muscle fibers for 7 days. On day 3 of incubation, E5 chick spinal cords were added to some of the cultures (+SC). Other cultures lacked innervation (–SC). IP3R1 was detected with a rabbit anti-IP3R1 antibody (+IP3R Ab) and a fluorescein-conjugated (green) secondary antibody. Control cultures lacked addition of the IP3R1 primary antibody (–IP3R Ab), but were incubated with the fluorescein-conjugated secondary antibody. Addition of spinal cords to muscle fiber cultures generates clusters of acetylcholine receptors in muscle fibers, which were detected by addition of rhodamine-conjugated (red) α-bungarotoxin. Bar, 50 μm.
IP3R inhibitor, 2-aminoethoxydiphenylborate (2-APB) or xestospongin D (Powell et al., 2001). Muscle fibers were then immunostained for fast MyHCs and slow MyHC2 with monoclonal antibodies F59 and S58, respectively (Fig. 3). As previously demonstrated (DiMario and Stockdale, 1997), all muscle fibers in vitro, whether of fast PM or slow MA origin, expressed a fast MyHC gene(s) and immunostained with F59. Non-innervated muscle fibers, whether of PM or MA origin, did not express the slow MyHC2 gene. Expression of the slow MyHC2 gene was not induced in non-innervated muscle fibers cultured in control medium or medium containing either 2-APB or xestospongin D. However, innervated MA muscles did express the slow MyHC2 gene and immunostained with S58. Expression of the slow MyHC2 gene in these fibers was independent of the presence of 2-APB or xestospongin D in the culture medium. Rather, expression of the slow MyHC2 gene in MA muscles was dependent on innervation. In contrast, innervated fast PM muscle fibers were resistant to slow MyHC2 gene expression. These results are in agreement with previously published data (DiMario and Stockdale, 1997; Jordan et al., 2003). However, innervated PM muscle fibers cultured in medium containing either 2-APB or xestospongin D did express the slow MyHC2 gene and immunostained with S58. These results indicate that inhibition of IP3R1 activity by 2-APB and xestospongin D in innervated fast PM muscle fibers elicits a fast-to-slow transition in muscle fiber type.

**Inhibition of IP3R1 activity reduces PKC activity**

Previous studies have shown that PKC activity is significantly reduced by inhibition of IP3R1 activity with 2-APB or xestospongin D.
IP3R1 activity regulates muscle fiber type

greater in fast PM muscle fibers relative to slow MA muscle fibers and that innervation reduces PKC activities in both fiber types (DiMario, 2001; DiMario and Funk, 1999). However, PKC activity in innervated PM muscle fibers was still greater than in innervated MA muscle fibers (Jordan et al., 2003). As reduced PKC activity has been correlated with slow MyHC2 gene expression (above references), we hypothesized that PM muscle fibers, induced to express the slow MyHC2 gene by treatment with 2-APB, exhibited significantly less PKC activity. To determine the effects of IP3R1 inhibition by 2-APB on PKC activity, innervated and non-innervated PM muscle fibers were incubated in control medium or medium containing 2-APB. PKC activities in the PM muscle fibers were then quantified (Fig. 4). Innervation of PM muscle fibers in control medium caused a 70% reduction in PKC activity. A similar 84% reduction in PKC activity occurred in non-innervated PM muscle fibers cultured in medium containing 2-APB. There was no significant difference between PKC activities in innervated muscle fibers cultured in control medium compared to non-innervated muscle fibers cultured in medium containing 2-APB. However, addition of 2-APB to culture medium of innervated PM muscle fibers significantly reduced PKC activity further. PKC activity in these muscle fibers was reduced by approximately 95% relative to non-innervated muscle fibers in control medium. These results indicate that IP3R1 activity in innervated and non-innervated PM muscle fibers stimulates PKC activity. Furthermore, as elevated PKC activity repressed slow MyHC2 gene expression (DiMario and Funk, 2001) and innervated PM muscle fibers cultured in medium containing 2-APB had decreased PKC activity and expressed the slow MyHC2 gene, these results suggest that IP3R1 activity represses slow MyHC2 gene expression in innervated PM muscle fibers.

Innervation and IP3R1 inhibition induce slow MyHC2 promoter activity

The slow MyHC2 promoter has been previously characterized (Jiang et al., 2004), and it is dependent on muscle fiber innervation for transcriptional activation. Innervation-dependent promoter activation was restricted to MA muscle fibers. The promoter was not activated in innervated PM muscle fibers (Jiang et al., 2004). To determine the effects of IP3R1 activity on transcriptional regulation of the slow MyHC2 gene, PM myogenic cultures were transfected with the DNA construct, 2279SM2Luc, containing the slow MyHC2 promoter driving a luciferase reporter gene (Fig. 5). Innervation or IP3R1 inhibition alone did not significantly increase slow MyHC2 promoter activity. Bars represent mean (±s.e.m.) fold increase in activity compared to levels in the control (n=6).

IP3R1 activity regulates NFAT-mediated transcription

Previous characterization of the transcriptional regulation of the slow MyHC2 promoter determined that MEF2 and NFAT binding sites were required for maximal promoter activity in innervated MA muscle fibers (Jiang et al., 2004). Mutation of the MEF2 binding site fully abrogated slow MyHC2 promoter activity.
activity, and mutation of the NFAT binding site partially reduced promoter activity. To determine whether IP3R1 activity regulates MEF2 or NFAT transcription factor activity, transcriptional reporter sensors containing the luciferase reporter gene transcriptionally regulated by multimerized MEF2 (MEF2Luc) or NFAT (NFATLuc) binding sites were transfected into PM myogenic cultures. Innervated and non-innervated muscle fibers were cultured in control medium or medium containing 2-APB. As shown in Fig. 6, MEF2-dependent transcriptional activity was increased approximately 5.5-fold by innervation, in agreement with previous findings (Jiang et al., 2004). Addition of 2-APB to culture medium did not significantly affect MEF2-mediated transcriptional activity in either non-innervated or innervated PM muscle fibers.

NFAT-mediated transcriptional activity was differentially regulated by innervation and 2-APB (Fig. 6). NFAT-mediated transcriptional activity increased by approximately 30% owing to innervation. However, inhibition of IP3R1 activity by 2-APB significantly reduced (55%) NFAT sensor activity in innervated PM muscle fibers. These results suggest that IP3R1 activity normally increases NFAT-mediated transcriptional activity in innervated muscle fibers.

**Western blot analyses of NFAT and MEF2**

Western blots of nuclear extracts from PM and MA muscle fiber cultures were performed with NFATc1 and MEF2A antibodies (Fig. 7). Nuclear extracts were prepared from innervated and non-innervated PM and MA muscle fibers cultured in either control medium or medium containing 100 µM 2-APB. NFATc1 was detected in nuclear extracts from both non-innervated PM and MA muscle fibers cultured in control medium and in medium containing 2-APB. However, the abundance of NFATc1 was reduced in nuclear extracts from PM and MA muscle fibers cultured in the presence of 2-APB. In nuclear extracts from innervated PM muscle fibers, the relative abundance of NFATc1 was also reduced by the presence of 2-APB. Nuclear extracts from innervated MA muscle fibers contained the greatest abundance of NFATc1. However, this was also reduced by the presence of 2-APB in the culture medium. These results are consistent with the NFAT sensor data of Fig. 6 in which 2-APB reduced NFAT-mediated transcriptional activity in both innervated and non-innervated PM muscle fibers.

**Discussion**

Skeletal muscle cells control release of calcium from intracellular stores by two receptor-mediated mechanisms. Activation of the RyR is a well-studied mechanism by which calcium is released from the SR as a high amplitude calcium spark. It has been shown that such calcium sparks inhibit NFAT nuclear localization (Gomez et al., 2002), and we have shown that inhibition of RyR1 activity enhances NFAT transcriptional activity and slow MyHC2 gene expression (Jordan et al., 2004). Calcium is also released from the SR via activation of IP3R1 by IP3 ligand binding. IP3R activation in many cell types leads to repetitive low amplitude (in relation to RyR-mediated calcium sparks) increases in intracellular calcium that are propagated as waves (Rooney et al., 1990; Lechleiter et al., 1991). In skeletal muscle cells, the slow wave of calcium release is blocked by the IP3R1 inhibitors 2-APB and xestospongin D (Powell et al., 2001).

To investigate the potential role of IP3R1 in the expression of muscle fiber type-specific genes, IP3R1 was immunodetected in innervated and non-innervated fast PM and slow MA muscle fibers in vitro. IP3R1 was detected in innervated and non-innervated PM and MA muscle fibers. However, a quantitative difference in the abundance of IP3R1 protein was detected on western blots. Innervated and non-innervated fast PM muscle fibers contained greater amounts of IP3R1 than innervated and non-innervated slow MA muscle fibers, respectively. The increased abundance of IP3R1 in fast PM muscle fibers may contribute to the total increased calcium release transient characteristic of fast muscle fibers.

The functional significance of IP3R1 activity on expression of the slow MyHC2 gene was tested by inhibition of IP3R1 activity in innervated PM and MA muscle fibers. Whereas innervation was sufficient to induce slow MyHC2 gene expression in MA muscle fibers (DiMario and Stockdale,
MEF2A nuclear abundance was not obviously affected by any of the fibers compared to the same cultures incubated in control medium. APB reduced NFAT abundance in nuclear extracts from all muscle conjugated secondary antibodies. Inhibition of IP3R1 activity by 2-APB reduced NFAT abundance in nuclear extracts from all muscle fibers compared to the same cultures incubated in control medium. MEF2A nuclear abundance was not obviously affected by any of the other conditions tested.

1997) (Fig. 3), inhibition of IP3R1 activity in conjunction with innervation was required for slow MyHC2 gene expression in fast PM muscle fibers. PM muscle fibers are normally resistant to innervation-induced slow MyHC2 gene expression. However, inhibition of IP3R1 activity relieved this repression. The specificity of IP3R1 inhibition by 2-APB was verified by use of the IP3R1-specific inhibitor, xestospongin D. Inhibition of IP3R1 activity by either 2-APB or xestospongin D induced an identical pattern of slow MyHC2 gene expression in response to innervation of PM muscle fibers. As IP3R1 activation causes a slow calcium wave release, it is reasonable to hypothesize that increased IP3R1 in PM muscle fibers generates an elevated calcium wave relative to MA muscle fibers. Furthermore, this elevated calcium wave may repress, or at least not be conducive to mechanisms that upregulate slow MyHC2 gene expression in innervated muscle fibers.

The IP3R1 agonist, IP3 and diacylglycerol (DAG) are generated by phospholipase C activity. DAG is an activator of PKC. In addition, calcium activates calcium-dependent PKC isoforms such as PKCα. We have previously shown that inhibition of PKC induced slow MyHC2 gene expression in innervated fast PM muscle fibers and that increased PKCα activity by over-expression repressed slow MyHC2 gene expression in innervated MA muscle fibers (DiMario and Funk, 1999; DiMario, 2001). The results presented here establish a functional link between IP3R1 activity and the previously published effects of PKC activity on slow MyHC2 gene expression. Innervated fast PM muscle fibers cultured in medium with the IP3R1 inhibitor, 2-APB, contained significantly lower PKC activity. This suggests that the increased IP3R1 in innervated fast PM muscle fibers elevates PKC activity relative to MA muscle fibers. Indeed, fast avian and mammalian muscle fibers have two- to threefold greater PKC activities than slow muscle fibers (DiMario and Funk, 1999; Donnelly et al., 1994). Therefore, we propose that the net calcium transient released by the combined activities of RyR1 and IP3R1 regulate expression of the slow MyHC2 gene in innervated muscle fibers. Fast PM muscle fibers have increased amounts of both RyR1 and IP3R1 relative to slow MA muscle fibers (Jordan et al., 2004) (our results). The increased calcium release channels in PM muscle fibers probably generate higher amplitude calcium transients emanating from both RyR1-mediated sparks and IP3R1-mediated waves. Inhibition of either calcium release mechanism de-represses slow MyHC2 gene expression in innervated fast PM muscle fibers.

Expression of the slow MyHC2 gene is transcriptionally regulated by NFATc1 and MEF2A (Jiang et al., 2004). Both NFAT and MEF2 have been proposed to be regulated by calcium-dependent phosphatase activity. Dephosphorylated NFAT is imported into muscle nuclei where it transcriptionally activates NFAT-dependent promoters (Chin et al., 1998; Crabtree, 2001). Similarly, it has been proposed that MEF2-dependent promoter activity is increased via dephosphorylation of MEF2 by calcium-dependent phosphatase activity (Wu et al., 2001). As the slow MyHC2 promoter in innervated fast PM muscle fibers was activated by IP3R1 inhibition, MEF2 and NFAT-dependent transcriptional activity was assessed. Innervation of fast PM muscle fibers significantly increased MEF2-mediated transcriptional activity as previously shown (Jiang et al., 2004; Wu et al., 2001). Inhibition of IP3R1 activity did not significantly affect MEF2-mediated transcription. However, NFAT-dependent transcription was reduced by IP3R1 inhibition in innervated PM muscle fibers. These results were corroborated by immunodetection of NFATc1 and MEF2A in nuclear extracts of muscle fibers in the presence of 2-APB. Inhibition of IP3R1 activity reduced NFAT nuclear localization whereas no detectable difference was observed for MEF2. The findings are in general agreement with the proposed mechanism of calcineurin-dependent dephosphorylation of NFAT. Low amplitude, long duration calcium transients are proposed to activate calcineurin leading to NFAT dephosphorylation and increased NFAT-dependent transcription. It is likely that IP3R1-mediated release of calcium waves increases NFAT activity, possibly via calcineurin-dependent dephosphorylation.

At first approximation, it is not clear how the NFAT-dependent slow MyHC2 gene is expressed in innervated fast PM muscle fibers in which IP3R1 activity is inhibited and NFAT transcriptional activity is reduced. Several considerations provide a more refined view of the role of NFAT in slow MyHC2 gene expression. First, although the slow MyHC2 promoter is dependent on NFAT-promoter interaction at NFAT binding sites, mutation of these sites abrogated NFAT binding but did not fully abrogate slow MyHC2 promoter activity in innervated slow MA muscle fibers (Jiang et al., 2004). Loss of NFAT-mediated transcriptional activity incrementally decreased slow MyHC2 promoter activity. Second, the slow MyHC2 promoter is dependent on MEF2A binding. Mutation of the MEF2A binding site reduced slow MyHC2 promoter activity to basal levels suggesting that MEF2A is a strong activator of this promoter. This was supported by a sixfold activation of MEF2A-dependent transcription in innervated PM muscle fibers compared to an approximate 0.3-fold increase in NFAT-mediated transcription. MEF2A-mediated transcription was not repressed by IP3R1 inhibition. Therefore, MEF2A retained its strong activation potential in innervated PM muscle fibers. Third, inhibition of

![Fig. 7. Western blot analyses of NFATc1 and MEF2A in muscle fiber nuclear extracts. Innervated (SC) and non-innervated PM and MA muscle fibers were cultured in control medium or medium supplemented with 100 μM 2-APB. Muscle fiber nuclear extracts were prepared, electrophoresed and immunoblotted. NFATc1 and MEF2A were detected with respective primary antibodies and HRP-conjugated secondary antibodies. Inhibition of IP3R1 activity by 2-APB reduced NFAT abundance in nuclear extracts from all muscle fibers compared to the same cultures incubated in control medium. MEF2A nuclear abundance was not obviously affected by any of the other conditions tested.](Image 66x627 to 281x719)
IP3R1 activity reduced PKC activity in innervated PM muscle fibers. As the slow MyHC2 gene is expressed in innervated fast PM muscle fibers in which either IP3R1 or PKC are inhibited, it is possible that IP3R1-dependent PKC activity regulates slow MyHC2 gene expression irrespective of NFAT transcription factor activity.

These results indicate that slow MyHC2 gene expression and muscle fiber phenotype are regulated by IP3R1 activity in innervated PM muscle fibers. Inhibition of IP3R1 activity caused slow MyHC2 gene expression, indicating that IP3R1 normally represses the slow muscle fiber phenotype in fast PM muscle cells. As IP3R1 releases calcium from the SR as a wave, the increased IP3R1 in innervated PM muscle fibers may generate a calcium wave that is insufficient to induce slow MyHC2 gene expression.

References


