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Repression of slow myosin heavy chain 2 gene expression in fast skeletal muscle fibers by muscarinic acetylcholine receptor and Gαq signaling

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Gene expression in skeletal muscle fibers is regulated by innervation and intrinsic fiber properties. To determine the mechanism of repression of *slow MyHC2* expression in innervated fast pectoralis major (PM) fibers, we investigated the function of the muscarinic acetylcholine receptor (mAchR) and Gαq. Both mAchR and Gαq are abundant in medial adductor (MA) and PM fibers, and mAchR and Gαq interact in these fibers. Whereas innervation of PM fibers was insufficient to induce *slow MyHC2* expression, inhibition of mAchR activity with atropine in innervated PM fibers induced *slow MyHC2* expression. Increased Gαq activity repressed *slow MyHC2* expression to nondetectable levels in innervated MA fibers. Reduced mAchR activity decreased PKC activity in PM fibers, and increased Gαq activity increased PKC activity in PM and MA fibers. Decreased PKC activity in atropine-treated innervated PM fibers correlated with *slow MyHC2* expression. These data suggest that *slow MyHC2* repression in innervated fast PM fibers is mediated by cell signaling involving mAchRs, Gαq, and PKC.

Introduction

Avian skeletal muscle is comprised of diverse fiber types expressing a variety of contractile protein isoform genes. Each fiber type expresses a unique repertoire of contractile protein genes that cumulatively determine the unique functional characteristics of each fiber type, such as contraction and relaxation rates and fatigability. Central to skeletal muscle fiber-type classification of fast and slow fiber types is expression of the fast and slow classes of myosin heavy chain (MyHC) isoform genes. The ATPase activities of the different MyHC isoforms directly correlate with muscle fiber contraction rates (Reiser et al., 1985, 1988).

For decades, it has been known that cues extrinsic to muscle fibers such as innervation, activity, and hormones influence patterns of contractile protein gene expression and corresponding muscle function. Studies using cross-reinnervation and denervation have demonstrated the substantive role of innervation in the regulation of whole muscle contractile activity and patterns of gene expression within individual muscle fibers (Pette and Vrbova, 1985). However, in contrast to the extensive body of work linking innervation with fiber type–specific gene expression, the cellular and molecular mechanisms that mediate the innervation-induced changes in contractile protein gene expression are only beginning to be elucidated.

In addition to extrinsic regulation of muscle fiber phenotype, avian skeletal muscle fibers also display patterns of MyHC gene expression that are intrinsically restricted to distinct myogenic cell lineages (Stockdale, 1997). These cell lineage–dependent, fiber type–specific patterns of fast versus slow MyHC gene expression were first observed in primary muscle fibers formed from embryonic myoblasts (Miller and Stockdale, 1986a; DiMario et al., 1993), but have since been demonstrated in secondary muscle fibers formed from fetal myoblasts as well (DiMario and Stockdale, 1997). Fetal chicken myoblasts appear in hypaxial musculature at approximately embryonic day 8 (ED8; Feldman and Stockdale, 1992). Continued proliferation and differentiation of fetal myoblasts through ED18 contributes to the formation of the majority of skeletal muscle fibers in the adult. The distribution pattern of secondary muscle fibers formed from fetal myoblasts and the fiber type–defining pattern of fast versus slow MyHC gene expression in vivo is reflective of the inner-

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vation independent pattern of MyHC gene expression in primary muscle fibers. All primary and secondary muscle fibers of the medial adductor (MA) muscle express fast MyHC genes and the slow MyHC2 gene (Page et al., 1992). All primary and secondary muscle fibers of the pectoralis major (PM) muscle (except the superior red strip) express only fast MyHC genes. Although MA and PM secondary muscle fibers differ in their expression of *slow MyHC2* in vivo, muscle fibers derived from fetal MA and PM myoblasts only express fast MyHC genes in vitro. However, muscle fibers formed from fetal slow MA myoblasts have been induced to express *slow MyHC2* by innervation from randomized spinal cord explants in vitro (DiMario and Stockdale, 1997). Furthermore, innervated muscle fibers formed from fast PM fetal myoblasts in vitro were repressed in their expression of *slow MyHC2*.

Functional innervation of skeletal muscle fibers causes fiber depolarization, release of intracellular calcium from the sarcoplasmic reticulum, and activation of calcium responsive biochemical cascades. A number of studies in a variety of cell types have correlated cell depolarization with changes in transcription factor activity. For instance, depolarization of pancreatic β cells activated nuclear factor κ B (Bernal-Mizrachi et al., 2002), and potassium depolarization of skeletal muscle cells resulted in phosphorylation of cycle adenosine monophosphate (cAMP)–responsive element (Jaimovich and Carrasco, 2002). Other recent evidence has indicated that free intracellular calcium amplitudes and transients characteristic of slow skeletal muscle fibers activates the protein phosphatase, calcineurin, which may then dephosphorylate members of the nuclear factor of activated T cell transcription factor family. Dephosphorylated nuclear factor of activated T cells can be imported into the nuclear compartment where they are proposed to activate transcription of genes indicative of the slow fiber phenotype, such as slow troponin I (Chin et al., 1998).

Cellular innervation also elicits signal transduction cascades initiated by cell surface receptors. These signaling cascades are often transduced from cell surface receptors to intracellular signaling molecules by heterotrimeric guanine nucleotide-binding proteins (G proteins). Skeletal muscle contains Gαq, a Gα isoform that is insensitive to pertussis toxin. Activated Gαq causes activation of phospholipase Cβ and subsequent generation of inositol triphosphate (IP3) and diacylglycerol (DAG; Taylor et al., 1991; Wilkie et al., 1991; Wu et al., 1992). In vertebrate cardiac tissue, Gαq is associated with the M1 muscarinic acetylcholine receptor (mAchR) and, in conjunction with calcineurin, may mediate signal transduction pathways leading to physiological responses, such as cardiac hypertrophy with accompanying changes in MyHC gene expression (Mende et al., 1998). Very little is known regarding the function of mAchRs and Gαq in skeletal muscle. Of the five types of identified mAchRs, type M1 AchR is present in skeletal muscle cells. Studies using the muscarinic antagonist, atropine, have demonstrated that mAchRs regulate glucose uptake in C_2C_{12} cells and that muscle fiber–associated mAchRs bind acetylcholine released from motor neurons (Welsh and Segal, 1997; Liu et al., 2002). Gαq-stimulated production of DAG and IP3 by PLC and release of calcium via intracellular cal-

cium release channels, such as the ryanodine and IP3 receptors, provide intracellular activators of PKC. Interestingly, several studies have implicated PKC activity in the depolarization and activity-dependent regulation of skeletal muscle genes such as the nicotinic AchR (nAchR), N-CAM, and N-cadherin (Klarsfeld et al., 1989; Hahn and Covault, 1992; Rafuse and Landmesser, 1996). In addition, expression of *slow MyHC2* is regulated by both innervation and PKC activity (DiMario and Funk, 1999; DiMario, 2001). Innervation-induced expression of *slow MyHC2* in MA muscle fibers is accompanied by decreased PKC activity. Furthermore, overexpression of PKC abrogates *slow MyHC2* expression in innervated MA muscle fibers.

Here, we examine the cellular mechanisms controlling innervation-induced expression of *slow MyHC2* in a fiber type–specific manner in the context of cell lineage restriction. Using an in vitro nerve–muscle coculture system, we demonstrate that mAchR and Gαq are associated in innervated fast and slow skeletal muscle fibers. mAchR and Gαq signaling increased PKC activities in PM and MA muscle fibers. Conversely, inhibition of mAchR activity in innervated fast PM muscle fibers resulted in fiber-type transition and expression of *slow MyHC2*. Furthermore, constitutively active Gαq repressed *slow MyHC2* expression in innervated slow MA muscle fibers.

Results

Based on previous results demonstrating that expression of *slow MyHC2* was regulated by PKC activity (DiMario and Funk, 1999), we hypothesized that upstream cellular signaling molecules such as the mAchR and Gαq may be present as potential regulators of PKC activity in PM and MA muscle fibers. To determine whether chicken PM and MA muscle express mAchR and Gαq, Western blots were performed on whole muscle cell extracts using M1 type mAchR and Gαq antibodies (Fig. 1). Both PM and MA contained readily detectable mAchR and Gαq in approximately equal amounts. These results are in agreement with studies of murine skeletal muscle in which Gαq and Gα11 mRNAs were present, and Gα14 and Gα15 mRNAs were absent (Strathmann and Simon, 1990; Wilkie et al., 1991).

To determine whether the mAchR and Gαq proteins interact in PM and MA muscle cells, myogenic cultures were transfected with the expression construct, GαqFLAG,
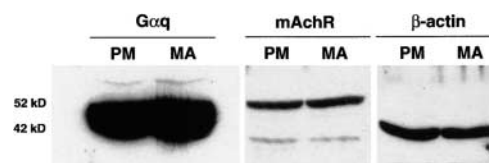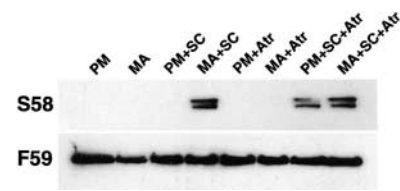

Figure 1. **Western blot analysis of Gαq and mAchR in PM and MA muscle.** Protein extracts from ED13 PM and MA muscles were prepared, electrophoresed, and blotted as described in Materials and methods. Blots were incubated with Gαq, mAchR, and β-actin–specific antibodies and HRP-conjugated secondary antibodies. Proteins were visualized by chemiluminescence. Both PM and MA contain readily detectable amounts of Gαq and mAchR. Equal amounts (100 μg) of PM and MA extracts were loaded as determined by BCA protein assays and detection of β-actin.
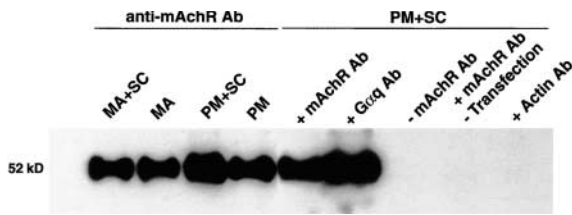
Figure 2. **Coimmunoprecipitation of mAchR and Gαg.** Extracts from innervated (+SC) and noninnervated PM and MA muscle fiber cultures expressing GαqFLAG were prepared. mAchR–Gαq protein complexes were immunoprecipitated using an anti-mAchR antibody. Controls using innervated PM muscle fibers (PM+SC) included direct immunoprecipitation of GαqFLAG with an anti-Gαq antibody, omission of the anti-mAchR antibody from the coimmunoprecipitation protocol, coimmunoprecipitation using extracts from nontransfected muscle fibers, and coimmunoprecipitation using an α-actin antibody. Co-immunoprecipitated mAchR–Gαq complexes were denatured, electrophoresed, and blotted. Gαq, coimmunoprecipitated with mAchR, was detected with an anti-FLAG antibody, HRP-conjugated secondary antibody and chemiluminescence.

Figure 4. **Western blot analysis of fast and slow MyHCs in innervated and noninnervated PM and MA muscle fiber cultures incubated in medium containing atropine.** ED13 PM and MA muscle fiber cultures were established. On day 3 of incubation, spinal cord (SC) explants were added to half the cultures and 200 μM atropine (Atr) was added to the medium of half the cultures. Fast and slow MyHCs were extracted from the cultures on day 7 of incubation, electrophoresed, blotted, and detected using mAbs F59 and S58, respectively. Culture extracts contained approximately equal amounts of fast MyHC. Slow MyHC2 was detected in extracts from innervated MA muscle fibers incubated with and without atropine. Slow MyHC2 was detected in extracts from innervated PM muscle fibers incubated in medium containing atropine.

which constitutively expressed wild-type Gαq coupled to a FLAG epitope. ED5 spinal cord explants were added to some of the plates of transfected cells to provide the muscle fibers with motor neuron innervation (DiMario and Stockdale, 1997). mAchR–GαqFLAG complexes were coimmunoprecipitated using an anti-mAchR antibody. Complexes were then denatured and Western blotted (Fig. 2). Gαq, which coimmunoprecipitated with mAchR, was detected with an anti-FLAG epitope antibody. GαqFLAG in transfected cultures of PM and MA with and without innervation coimmunoprecipitated with the mAchR. GαqFLAG was not detected in extracts from nontransfected cells or in extracts carried through the coimmunoprecipitation procedure without the mAchR antibody. To determine whether the mAchR–Gαq interaction in transfected cells was specific, protein extract was incubated with α-actin antibody. No GαqFLAG was detected in protein extracts immunoprecipitated with α-actin antibody. Therefore, Gαq and

mAchR interact in both innervated and noninnervated PM and MA muscle cells.

We hypothesized that mAchR activity regulated *slow MyHC2* expression in innervated muscle fibers. To test this hypothesis, innervated and noninnervated PM and MA muscle fiber cultures were incubated in medium containing 200 μM atropine, an antagonist of mAchR activity. Muscle fibers were then immunostained with mAbs F59 and S58, which specifically recognize fast and slow MyHC isoforms, respectively (Fig. 3). Consistent with previous findings (DiMario and Stockdale, 1997), innervation alone induced expression of *slow MyHC2* in MA muscle fibers, but not in PM muscle fibers. Noninnervated PM and MA muscle fibers incubated in medium containing 200 μM atropine also did not express *slow MyHC2*. However, both PM and MA muscle fibers expressed *slow MyHC2* in the presence of innervation and atropine. Similar qualitative results were obtained using medium containing atropine at concentrations of 100 and 500 μM (unpublished data).
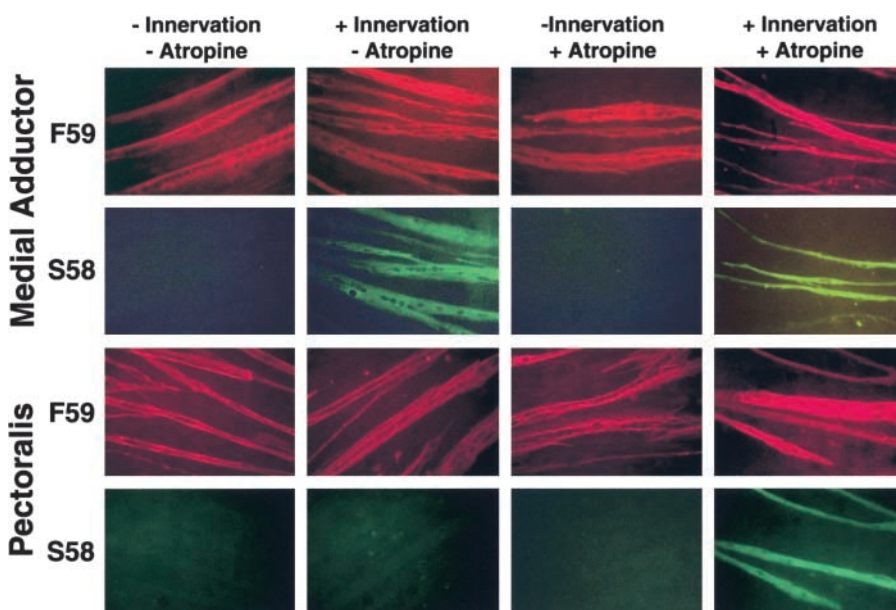
Figure 3. **Effects of innervation and atropine on *slow MyHC2* expression.** PM (pectoralis) and MA (medial adductor) muscle fiber cultures were prepared as described in Materials and methods. On day 3 of incubation, ED5 spinal cord explants were added to half of the cultures to provide innervation of muscle fibers. 200 μM atropine was also added to the culture medium of half the cultures on day 3 of incubation. On day 7 of incubation, cells were fixed and immunostained for fast MyHC and slow MyHC2 with mAbs F59 and S58, respectively, followed by fluorochrome-conjugated secondary antibodies. *slow MyHC2* expression was detected in innervated MA muscle fibers with and without atropine. *slow MyHC2* expression was detected in innervated PM muscle fibers incubated in medium containing atropine.

Immunostaining results were confirmed by Western blot analysis (Fig. 4). MyHC preparations of innervated and noninnervated PM and MA muscle fiber cultures incubated in control medium or medium containing 200 μM atropine were blotted and detected with F59 and S58 antibodies. Slow MyHC2 was present in innervated MA muscle fiber cultures incubated in medium with and without atropine. Slow MyHC2 was also detected in extracts from innervated PM muscle fiber cultures incubated in medium containing atropine. It is currently not known whether the observed doublet bands are due to degradation of slow MyHC2 or detection of slow MyHC3, a cardiac slow MyHC isoform transiently expressed in skeletal muscle and recognized by S58 (Nikovits et al., 1996). These results indicate that innervation is required for *slow MyHC2* expression in both PM and MA muscle fibers. Furthermore, mAchR activity in innervated PM muscle fibers normally represses *slow MyHC2* expression to nondetectable levels based on immunostaining.

Based on the interaction of the mAchR and Gαq, we hypothesized that mAchR activity–dependent repression of *slow MyHC2* expression was mediated by Gαq signaling. To test this hypothesis, a constitutively active, GTPase-deficient Gαq expression construct, CMVGαqQ209LFLAG, was generated yielding a single amino acid substitution (Q209→L209) and transfected into MA myogenic cultures. Similar mutations have resulted in constitutively active Gαq (Wu et al., 1992; Qian et al., 1993). As shown above (Figs. 3 and 4), MA muscle fibers expressed *slow MyHC2* when innervated and, therefore, these muscle fibers provide the most appropriate basis for forced repression of *slow MyHC2* expression. Transfected muscle fibers were identified with an anti-FLAG epitope antibody, and fast and slow MyHC2 were detected with F59 and S58 antibodies, respectively (Fig. 5). In addition, innervation of muscle fibers was visualized by detection of nAchR clusters using rhodamine-conjugated α-bungarotoxin. Whereas innervated, nontransfected MA muscle fibers expressed *slow MyHC2* (Fig. 5 A), innervated MA muscle fibers expressing GαqQ209LFLAG did not immunostain with S58 (Fig. 5 B). No muscle fibers containing detectable amounts of GαqQ209LFLAG expressed *slow MyHC2*. In contrast, expression of wild-type GαqFLAG in innervated MA muscle fibers did not repress *slow MyHC2* expression. Importantly, expression of GαqQ209LFLAG did not prevent MA muscle fiber innervation as indicated by clusters of nAchRs. In this culture system, these clusters are only evident when muscle fibers are innervated by spinal cord explants (DiMario and Stockdale, 1997). PM muscle fibers, whether innervated and/or transfected with CMVGαqQ209LFLAG, did not express *slow MyHC2* as determined by immunostaining (unpublished data). Therefore, Gαq activity repressed *slow MyHC2* expression in innervated MA muscle fibers.

We have shown previously that PKC activity regulates expression of *slow MyHC2* (DiMario and Funk, 1999). Inhibition of PKC activity induced *slow MyHC2* expression, and overexpression of PKC repressed *slow MyHC2* expression. Therefore, we hypothesized that the regulation of *slow MyHC2* by mAchR and Gαq activities was mediated by PKC activity. To determine whether mAchR and Gαq signaling controlled PKC activities in PM and MA muscle fibers, PKC activities were directly determined in cytoplasmic and membrane fractions obtained from PM and MA muscle
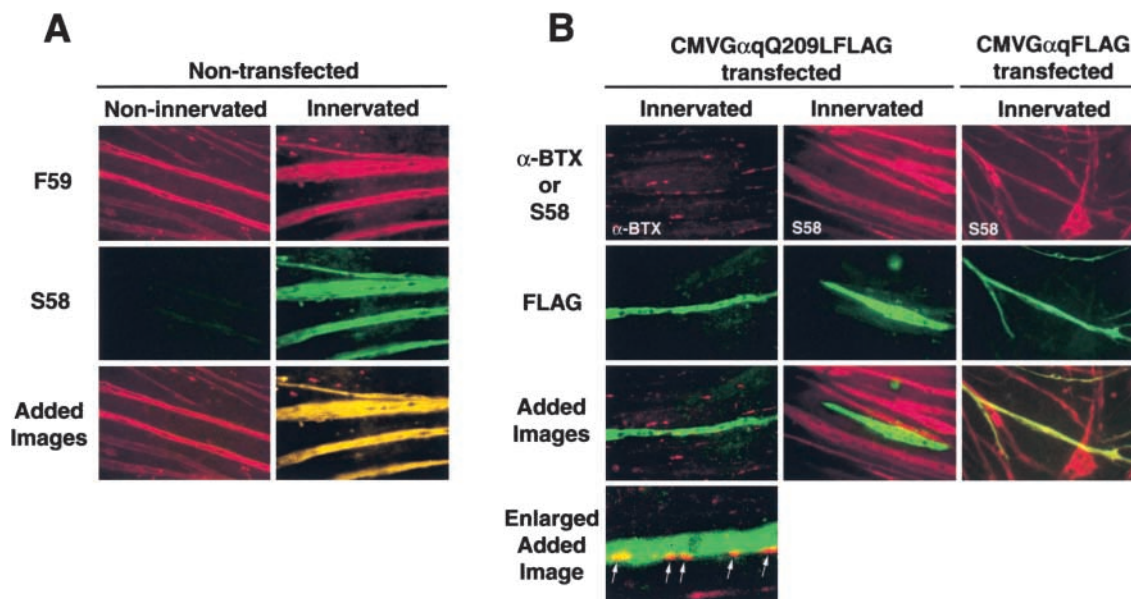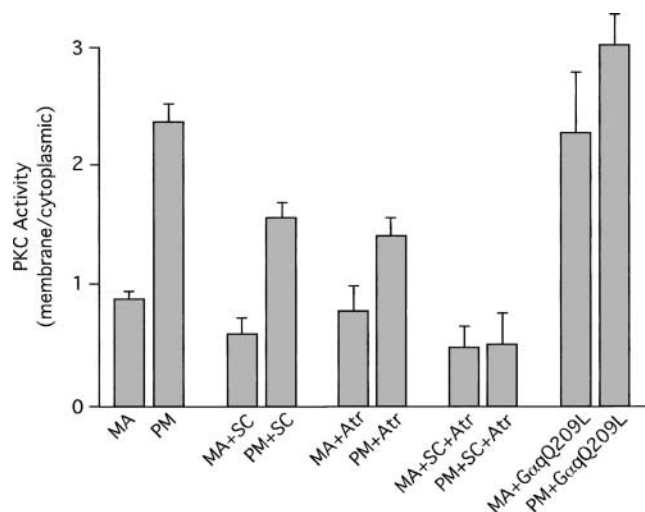

Figure 5. **Repression of *slow MyHC2* expression by Gαq activity.** MA myogenic cultures were established and some of the muscle fibers were innervated by spinal cord explants. Before addition of spinal cord explants, MA myogenic cultures were also transfected with either CMVGαqFLAG encoding wild-type Gαq or CMVGαqQ209LFLAG, which constitutively expresses GTPase-deficient, active Gαq. On day 7 of incubation, cells were fixed and immunostained with mAbs F59 and S58 to detect fast MyHCs and slow MyHC2, respectively, followed by fluorochrome-conjugated secondary antibodies. Clusters of nicotinic AchRs (arrows), indicative of innervated muscle fibers, were detected by incubation of the cells in medium containing rhodamine-conjugated α-bungarotoxin (α-BTX) before fixation. Transfected muscle fibers were identified by immunostaining with an anti-FLAG epitope antibody and an FITC-conjugated secondary antibody. Images were added digitally using Photoshop 5.0.

fiber cultures (Fig. 6). These cultures were generated by incubation of noninnervated and innervated PM and MA muscle fibers in control medium and/or in medium containing 200 μM atropine. In addition, cytoplasmic and membrane fractions were obtained from PM and MA muscle fiber cultures transfected with CMVGαqQ209LFLAG. Membrane versus cytoplasmic PKC activities were determined to quantitate membrane-associated active PKC in the cultures. Noninnervated PM muscle fibers contained 2.5-fold more PKC activity than noninnervated MA muscle fibers. This is in agreement with previous studies examining PKC activities in avian and mammalian fast versus slow type muscles (Donnelly et al., 1994; DiMario and Funk, 1999). Innervation of both PM and MA muscle fibers significantly reduced PKC activities. Innervation of PM muscle fibers resulted in an ∼33% reduction in membrane versus cytoplasmic PKC activity. PM muscle fibers incubated in medium containing atropine also yielded reduced PKC activity by 41%. Combination of innervation of PM muscle fibers and incubation in medium containing atropine resulted in a significant, additive inhibition of PKC activity. PKC activities in MA muscle fibers incubated in control medium or medium containing atropine were not significantly different. Last, both PM and MA muscle fiber cultures expressing constitutively active Gαq yielded significantly increased PKC activities. These results indicate that direct modulation of mAchR and Gαq activities affects downstream PKC activities, particularly in PM muscle fibers. Reduced mAchR/Gαq signaling resulted in reduced PKC activity, and increased mAchR/Gαq signaling increased PKC activity.

Figure 6. **PKC activities are modulated by innervation and atropine.** ED13 PM and MA muscle fiber cultures were established. Some muscle fiber cultures were cocultured with ED5 spinal cord explants (+SC) beginning on day 3 of incubation. Other cultures were incubated in medium containing 200 μM atropine (+Atr) from days 3–7 of incubation. Some cultures received both innervation and atropine (+SC+Atr). Yet other cultures were transfected 24–48 h after plating with CMVGαqQ209LFLAG and incubated until day 7 without innervation or atropine. On day 7 of incubation, cells were scraped from the culture dishes and membrane versus cytoplasmic fractions were prepared. Protein contents of each fraction were determined by BCA protein assay. PKC activities in each fraction were determined using a PKC assay kit and were normalized to protein content. Data are expressed as membrane associated PKC activity normalized to cytoplasmic PKC activity. Bars indicate the mean of three independent experiments ± SEM. Significant differences in means were determined by t test with $P < 0.05$.

Discussion

Phenotypic differences among avian and mammalian skeletal muscle fiber types are regulated by a variety of cellular and molecular mechanisms. These mechanisms are often initiated by factors extrinsic to the muscle fibers themselves, such as innervation. These extrinsic factors potentially elicit numerous alterations in muscle specific and nonmuscle specific patterns of gene expression that are in concert with the altered contractile and metabolic needs of the muscle fiber. However, this muscle fiber plasticity is often tempered by muscle fiber specific restrictions to altered patterns of gene expression. A combinatorial perspective of extrinsic and intrinsic parameters acting together to determine muscle fiber phenotype is easily observed in nerve–muscle fiber type–specific cocultures. We have previously shown that muscle fibers of slow muscle origin (MA) are induced to express *slow MyHC2* and thereby convert their fiber type by innervation in vitro. However, muscle fiber type–repression of *slow MyHC2* expression occurred in identically innervated muscle fibers of fast muscle origin (PM; DiMario and Stockdale, 1997).

Here, we have begun to identify the mechanism by which expression of *slow MyHC2* and the slow muscle fiber phenotype is repressed in innervated fast muscle fibers. Based on previous findings that inhibition of PKC activity in noninnervated MA muscle fibers was sufficient to induce *slow MyHC2* expression, that innervated and noninnervated PM muscle fibers contained significantly greater PKC activity, and that innervation of muscle fibers decreased PKC activ-

ity, we hypothesized that a signaling cascade mediating innervation with PKC activity at least partly defined the intrinsic repression of *slow MyHC2* expression in innervated fast PM muscle fibers.

PKC activity is regulated via activity of PLC, which generates DAG and IP3. In turn, PLC is activated from cell surface receptors via the heterotrimeric guanine nucleotide binding protein Gαq. To determine whether the cell surface receptor, mAchR, and Gαq were present in PM and MA muscle fibers, Western blots were performed. Both mAchR and Gαq were readily detectable in PM and MA muscle fibers. Furthermore, mAchR and Gαq were coimmunoprecipitated, indicating a physical, and potentially functional, interaction. These results are in agreement with independent investigations that demonstrated expression of Gαq in skeletal muscle (Wilkie et al., 1991) and that functional mAchRs are present in cultured skeletal muscle fibers (Reyes and Jaimovich, 1996). Furthermore, mAchRs can be activated by acetylcholine from neuromuscular junctions (Welsh and Segal, 1997).

Functionality of mAchRs with regard to *slow MyHC2* expression was examined in PM and MA muscle fibers. Innervated and noninnervated PM and MA muscle fibers were incubated in medium containing 200 μM atropine, an antagonist of mAchRs. Based on immunostaining for fast MyHCs and slow MyHC2, atropine had no effect on MA muscle fibers. However, incubation of innervated PM mus-

cle fibers in medium containing atropine induced *slow MyHC2* expression and resulted in a fiber-type transition. This is the first report documenting avian fast muscle fiber-type transition to a slow muscle fiber phenotype.

Gαq transduces activation of mAchRs to downstream targets, such as PLC. To determine whether Gαq signaling regulates *slow MyHC2* expression, a constitutively active, GTPase deficient mutation of Gαq was generated. Identical mutations of Gαq Q209→L209 resulted in unregulated active Gαq (Qian et al., 1993; Mende et al., 1998). Expression of constitutively active Gαq in innervated MA muscle fibers repressed *slow MyHC2* expression, whereas expression of wild-type Gαq had no detectable effect on *slow MyHC2* expression. Activated Gαq did not negate innervation of MA muscle fibers. Therefore, repression of *slow MyHC2* expression was due to Gαq activity and not due to lack of innervation. Together, these results indicate that cell signaling via mAchR and Gαq represses *slow MyHC2* expression. This mechanism appears to be normally operative in PM muscle fibers since inhibition of mAchR activity in innervated PM muscle fibers resulted in derepression of *slow MyHC2* expression. Furthermore, activation of the mAchR–Gαq pathway in innervated, normally slow MA muscle fibers repressed *slow MyHC2* expression and yielded a slow to fast fiber-type transition.

Based on previous findings indicating that increased PKC activity repressed *slow MyHC2* expression (DiMario, 2001), we hypothesized that repression of *slow MyHC2* expression by activated Gαq would be accompanied by increased PKC activity. Measurements of PKC activities in innervated and noninnervated PM and MA muscle fibers incubated in medium with and without atropine indicated that decreased PKC activity was correlated with *slow MyHC2* expression. As described previously (DiMario, 2001), fast muscle fibers contain greater PKC activity than slow fibers, whether innervated or not. Interestingly innervation reduced PKC activity in both PM and MA muscle fiber types, although PKC activity in innervated PM was greater than the activities in innervated and noninnervated MA muscle fibers. The mechanism by which innervation reduces PKC activity is currently not known. PKC activity in noninnervated PM muscle fibers was also reduced by incubation in medium containing atropine. PKC activity in MA muscle fibers was unaffected by atropine. The reduction in PM muscle fiber PKC activity is likely due to inhibition of a basal level of mAchR–Gαq activation of PKC. Importantly, addition of atropine to innervated PM muscle fibers reduced PKC activity to levels that were not significantly different from activities in innervated MA muscle fibers with and without atropine. These three experimental conditions (MA plus innervation, MA plus innervation plus atropine, and PM plus innervation plus atropine) resulted in *slow MyHC2* expression.

Regulation of muscle fiber gene expression by mechanisms initiated by electrical and contractile activities and mediated by PKC activity have been demonstrated for expression of nAchR, N-CAM, and N-cadherin (Klarsfeld et al., 1989; Hahn and Covault, 1992; Rafuse and Landmesser, 1996). Based on the evidence that innervation reduces PKC activity in MA and PM muscle fibers and that

inhibition of mAchR activity is required for *slow MyHC2* expression in innervated PM muscle fibers, it is proposed that multiple signaling pathways regulate PKC activity and ultimately *slow MyHC2* expression in PM muscle fibers. Reduction of PKC activity in innervated PM muscle fibers is blunted by concomitant activation via mAchR–Gαq signaling. This intermediate reduction in PKC activity is insufficient to de-repress *slow MyHC2* expression.

It is not known if mAchR–Gαq activation of PKC occurs in innervated MA muscle fibers. Similar to PM muscle fibers, MA muscle fibers contain readily detectable levels of both mAchR and Gαq. mAchR and Gαq were coimmunoprecipitated in extracts of innervated and noninnervated MA muscle fibers. Yet, incubation of MA muscle fibers in medium containing atropine did not significantly reduce PKC activity as it did in PM muscle fibers. Furthermore, MA PKC activity did significantly increase due to constitutive expression of active Gαq. Therefore, mAchR–Gαq signaling may be uncoupled from modulation of PKC or repressed by an impinging signaling circuit in MA muscle fibers.

In total, these results suggest that innervation of MA muscle fibers causes a reduction in PKC activity and that, as shown previously (DiMario, 2001), this is sufficient to induce a signaling cascade leading to *slow MyHC2* expression. In PM muscle fibers, innervation causes a reduction in PKC activity, but this reduction is tempered by cell signaling via mAchR–Gαq, which tends to increase PKC activity. This modulation of PKC activity is evident in the partial reduction of PKC activity in innervated PM muscle fibers and in fibers exposed to atropine. These declines yield PKC activities that are significantly greater than MA muscle fibers under any nontransfected experimental condition. Combination of innervation and atropine exposure reduced PKC activity in PM muscle fibers to levels that were not significantly different than the PKC activities in MA muscle fibers, and it was this experimental condition (spinal cord plus atropine) that resulted in *slow MyHC2* expression in formerly fast PM muscle fibers.

Materials and methods

Cell culture and transfections

Myoblasts were isolated from ED13 PM and MA muscles and cultured in 35-mm collagen coated dishes as described previously (O'Neill and Stockdale, 1972; Miller and Stockdale, 1986b). Cell culture medium consisted of 10% horse serum (Hyclone), 5% chick embryo extract, in Ham's F-10 supplemented with 1.1 mM $CaCl_2$, 2 mM glutamine, and penicillin/streptomycin/Fungizone (GIBCO BRL). Medium was changed every other day. To obtain nerve–muscle cocultures, ED5 chick spinal cords were dissected, minced, and placed into the myogenic cultures on day 3 of incubation as described previously (DiMario and Stockdale, 1997). Some cultures were incubated in medium containing atropine sulfate (Sigma-Aldrich) at final concentrations of 100, 200, and 500 μM. Myogenic cultures were transfected on day 2 of incubation. 4 μg/dish CMVGαqFLAG and 4 μg/dish CMVGαqQ209LFLAG were transfected using Lipofectamine Plus reagent (Life Technologies).

Immunofluorescence

Before fixation, some cultures were incubated in 100 nM rhodamine–conjugated α-bungarotoxin (Molecular Probes) for 1 h at 37°C to visualize nAchR clusters. Cultures were washed twice with PBS and fixed in 100% ethanol for 5 min. Cells were then incubated in 5% horse serum and 2% BSA in PBS for 1 h at room temperature. Muscle fiber and nerve–muscle cultures were immunostained for fast MyHCs and slow MyHC2 using

mAbs F59 and S58, respectively, and diluted 1:10 in blocking solution for 1 h at room temperature. The specificities of these antibodies have been established previously (Crow and Stockdale, 1986). Cells were washed three times with PBS. F59 and S58 antibodies were detected by incubating fluorochrome-conjugated anti–mouse IgG (Vector Laboratories) and anti–mouse IgA (Southern Biotechnology Associates, Inc.) secondary antibodies, respectively, for 1 h at room temperature. Expression of GαqFLAG and GαqQ209LFLAG was immunodetected using an anti-FLAG epitope antibody (Sigma-Aldrich) diluted 1:1,000 in blocking solution for 1 h at room temperature. Cells were washed three times with PBS and coverslipped.

Coimmunoprecipitation and Western blotting

ED13 PM and MA cultures were transfected with CMVGαqFLAG on day 2 of incubation. ED5 spinal cord explants were added to half of the cultures on day 3 of incubation. On day 7 of incubation, cultures were washed twice with PBS and 1ml of dithiobis (succinimidylpropionate; Pierce Chemical Co.) diluted to 2 mM in PBS was added to each plate for 1 h at room temperature after which 1 M Tris, pH 7.5, was added to a final concentration of 20 mM for 15 min at room temperature. Cells were scraped in 750 μl RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and homogenized by 10 passages through a 22-gauge needle. All steps were performed at 4°C. The extracts were spun in a centrifuge (Eppendorf) for 10 min at 14,000 rpm. 1 μg nonspecific rabbit IgG was added to each sample and incubated for 30 min. Protein A/G–agarose beads (Santa Cruz Biotechnology, Inc.) were added to each sample and incubated for 1 h. The beads were pelleted at 2,500 rpm for 1 min and the supernatants were transferred to new tubes. 2 μg anti-mAchR antibody (Santa Cruz Biotechnology, Inc.) was added to the samples (except control samples) and incubated for 4 h. Control samples were incubated with 2 μg anti-Gαq (Santa Cruz Biotechnology, Inc.) and anti–α-actin (Sigma-Aldrich) antibodies. Protein A/G–agarose beads were added and incubated overnight. The agarose beads were washed three times by pelleting the beads at 2,500 rpm for 1 min and resuspending them with cold RIPA buffer. Denaturing electrophoresis sample buffer was added to the pelleted beads and heated to 70°C for 10 min. Protein extracts were run in a 10% SDS polyacrylamide gel and Western blotted. The blot was incubated in 2% nonfat dry milk, 0.05% Tween 20 in 1 × PBS for 1 h at room temperature, and with anti-FLAG antibody (Sigma-Aldrich) diluted 1:1,000 in blocking solution for 1 h. The blot was washed three times with 0.05% Tween 20 in PBS and incubated in HRP-conjugated anti–mouse IgG (Santa Cruz Biotechnology, Inc.) diluted 1:1,000 in blocking solution for 1 h. The blot was washed as before and incubated in Supersignal West Pico Chemiluminescent substrate (Pierce Chemical Co.) before X-ray film exposure.

Western blots of mAchR and Gαq were similarly performed. ED13 PM and MA muscles were dissected and homogenized in 25 mM Tris, 1 mM EDTA, 50 mM NaCl, and 0.5% Triton X-100, pH 7.2. Each extract was electrophoresed in a 10% SDS polyacrylamide gel and blotted. Blots were incubated in anti-mAchR, anti-Gαq, and β-actin antibodies (Santa Cruz Biotechnology, Inc.) diluted 1:500 and processed as above (previous paragraph). For Western blots of MyHCs, innervated and noninnervated PM and MA muscle fiber cultures were scraped and homogenized on day 7 of incubation in 20 mM KCl, 2 mM K_2HPO_4, and 2 mM EGTA, pH 6.8. Extracts were incubated on ice overnight and centrifuged at 10,000 rpm in a rotor (model SS-34; Sorvall) for 20 min. The pellets were washed twice in homogenization buffer. Myosin was extracted from the pellets by resuspending the pellets in 80 mM $Na_4P_2O_7$, 2 mM $MgCl_2$, and 2 mM EGTA, pH 9.6. The extracts were incubated on ice for 2 h and centrifuged at 10,000 rpm for 20 min. Supernatants were collected and protein content was determined using a BCA protein assay (Pierce Chemical Co.). MyHCs were electrophoresed and blotted as above (previous paragraph). MyHCs were detected with F59 and S58 mAbs diluted 1:10 in blocking solution, followed by HRP-conjugated secondary antibodies as above (previous paragraph).

DNA constructs

Mouse Gαq cDNA, provided by M.I. Simon (California Institute of Technology, Pasedena, CA; Strathmann and Simon, 1990), was amplified using the following oligonucleotides: GATCGAAAGCTTGACCAGATTGTACTC-CTTC and GATCGAGCGGCCGCGCGAGGCACTTCGGAAGA. The PCR product was gel purified, digested with HinDIII and NotI restriction enzymes, and cloned into the CMVTAG4a expression vector (Stratagene). Correct, in-frame cloning relative to the FLAG epitope was verified by DNA sequencing. The following mutagenic oligonucleotides were used to PCR amplify the CMVGαqFLAG template DNA: GGTCGATG-TAGGGGGC<u>CTA</u>AGGTCAGAGAGAAG and CTTCTCTCTGACCT<u>TAG</u>-GCCCCCTACATCGACC. These oligonucleotides generated a point muta-

tion (underlined codon) at glutamine 209→leucine 209 rendering Gαq GTPase deficient and constitutively active (Qian et al., 1993). Mutagenesis was confirmed by DNA sequencing.

PKC assay

On day 7 of incubation, cultures of PM and MA muscle fibers were washed once with PBS and homogenized in cold 25 mM Tris, 1 mM EDTA, and 50 mM NaCl, pH 7.2, using a Dounce homogenizer type B. Extracts were spun in a centrifuge (Eppendorf) at 4°C for 10 min at 14,000 rpm. Supernatants were saved as cytoplasmic extracts. Pellets were resuspended in homogenization buffer containing 0.5% Triton X-100 and set on ice for 30 min. The extracts were spun for 10 min at 14,000 rpm and the supernatants were saved as membrane fractions. Protein concentrations were determined using a BCA protein assay reagent (Pierce Chemical Co.). PKC activities were determined using a PKC assay kit (Upstate Biotechnology) including inhibitors of PKA and CaM-dependent kinases.

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