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Control of myogenic factor genes by the membrane depolarization/protein kinase C cascade in chick skeletal muscle

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Myogenic factor genes were found to respond differentially to electrical stimulation of denervated chick skeletal muscle. Myogenin gene activity declined rapidly ($t_{1/2}$: ~2 min), comparable to the rate of acetylcholine receptor (AChR) gene inactivation, while other myogenic bHLH genes either lost activity more slowly (MyoD) or not at all (myf5, herculin). Protein kinase C (PKC) is known to couple membrane activity to AChR gene inactivation; myogenin gene transcription was also rapidly blocked by the PKC activator PMA, whereas electrostimulation remained without effect on myogenin gene activity in muscle that was either exposed to the kinase inhibitor staurosporine or chronically treated with PMA to deplete PKC. These results attest to a special role for myogenin in the activation of AChR genes in denervation supersensitivity.

Excitation–transcription coupling; Protein kinase C; Myogenin; Acetylcholine receptor; Chick skeletal muscle

1. INTRODUCTION

Adult skeletal muscle is plastic, i.e. capable of changing its phenotype in response to suitable stimuli. Among the changes in protein composition that can occur those affecting myofibrillar structures, energy metabolism, and excitability have been most thoroughly studied [1–4]. The neural influence on the expression of acetylcholine receptor (AChR) in particular has been investigated [5–7]. Promoters of all receptor subunit-encoding genes investigated so far contain MyoD binding sites ('E boxes') and are activated by myogenic factors [8–17]. That these factors may regulate expression of the γ isoform of the receptor not only during differentiation of myogenic cells but also during adaptive changes in adult muscle, is attested to by the presence of E boxes in activity response elements [18–20] and the effect of denervation and electrical stimulation on the levels of mRNAs coding for myogenin and MyoD, two prominent members of the family [21–23].

We have previously shown that the transcriptional activity of receptor subunit genes rises by approximately an order of magnitude upon the denervation of chick skeletal muscle [24,25] and that electrical stimulation leads to rapid inactivation of these genes [25]. An analysis of mRNA levels revealed that of the myogenic factors (MyoD, myogenin, myf5, and herculin), only myogenin mimicked the behavior of receptor subunits prompting us to propose a regulatory scheme in which autoactivation of myogenin plays a central role [23]; we have also shown that protein kinase C (PKC) couples membrane electrical activity to inhibition of AChR genes [25]. Consequently, if one or the other myogenic factor is assumed to control receptor genes in vivo, one should expect it to be targeted by PKC as well. Here we show that, among myogenic factors, myogenin is unique in that the denervation-induced activity of the gene coding for it is rapidly and completely blocked by signals from the depolarizing sarcolemma, and that these signals are mediated by PKC.

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2. MATERIALS AND METHODS

2.1. *Animal experiments*

Sciatic nerve section and electrical stimulation were performed on 4-day Leghorn chicks (Hall's Brothers Hatchery, North Brookfield, MA) as described previously [25]. Animal care was in accordance with institutional guidelines.

2.2. *Transcription elongation analysis*

Nuclei were isolated, and transcript elongation was carried out with 10^7 freshly prepared or liquid nitrogen-stored nuclei, using 150 μCi [^{32}P]UTP, as described previously [25], except that NaOH treatment was omitted and unlabeled UTP was added to a final concentration of 150 μM. Nascent transcripts were purified through a Sephadex G-50 spun column, followed by TCA and ethanol precipitations. For hybridization, aliquots containing a constant amount of radiolabel (10^6 cpm) were incubated with ~0.1 μg of antisense RNA probe at 52°C; RNase A digestion and electrophoretic analysis followed the procedure of Melton et al. [27]. A detailed description of the ribonuclease protection version of the run-on assay is to be published elsewhere. Results were quantified using a Beta scanner (Ambis, San Diego, CA) and visualized by autoradiography. Riboprobe templates for chicken myogenic factors were obtained as described previously. Probes for CMD1, the chicken homolog of MyoD (protected region: 102 nt), and for chicken myogenin (protected sequences: 200 and 390 nt) were derived from cDNA libraries, while short sequences corresponding to the HLH region of chicken myf5 and herculin (protected region: 194 nt) were obtained with the PCR from genomic DNA using

suitable primers [23]. The 399-bp *Hin*dIII–*Eco*RI genomic fragment of the chicken AChR α-subunit gene, containing exon 7 and flanking intron sequences [26], was cloned into the Bluescript plasmid pSK− in order to generate riboprobe. CTF4 probe was generated from a 171-bp PCR fragment derived from the coding region [28] and cloned into pKSII+; the probe protects primary transcripts of 50 and 121 nt. The 3′ end of the chicken c-*fos* cDNA was deleted by restricting pTZ19R/ch c-*fos* with *Bam*HI; the resulting plasmid was linearized with *Eco*RI, and the remaining 5′ fragment transcribed with T7 (protected region: 550 nt). The 3′ end of the chicken myosin light chain cDNA was removed by cutting MLC1f with *Sst*I; an antisense probe was prepared by linearizing the plasmid and transcribing with SP6 (protected region: 180 nt).

2.3. *Protein kinase C assay*

Nuclear high salt extracts were chromatographed on DEAE-cellulose. The fraction eluting between 100 and 400 mM NaCl was collected, and protein determined by the Bradford procedure; aliquots containing 1 μg of protein (ca. 5% of sample) were assayed for PKC as described previously [25], except that diolein was omitted from the incubation. The assay cocktail contained 20 mM Tris-HCl pH 7.5; 5 mM magnesium nitrate; and 0.2 mg/ml histone type III (Sigma). When included, final concentrations of supplements in the incubation mixture were: calcium chloride, 1 mM; phosphatidyl serine (Folch fraction III from bovine brain, Sigma), 40 μg/ml; and phorbol 12-myristate 13-acetate (PMA) (Sigma), 5 μM.

3. RESULTS

Fig. 1 shows transcription activities in innervated, denervated, and short-term stimulated muscle for all myogenic factors and several control genes. Myogenin gene activity increases ~16-fold upon denervation and drops ~20-fold upon 2 h of electrostimulation. Of the other factor genes, MyoD responds somewhat to these treatments, but much less than myogenin, approximately doubling in activity after denervation and returning to control (innervated) levels in stimulated muscle; no pronounced changes in the activities of the genes coding for myf5 and herculin are observed. A strong response of AChR subunit genes to membrane activity has been described previously [25]. Here results of the analysis of the α-subunit gene activity are included; they are very similar in rate and extent to the changes in the activity of the myogenin gene. The gene coding for the E protein CTF4 [28], which might function as a dimerization partner of a myogenic protein and thereby determine its activity, was also investigated, but no change in activity was seen (this finding does not rule out the possible limitation of maximal AChR gene transcription rate by the E protein). Similarly, the activity of the MLC gene is little affected by the experimental manipulations. The c-*fos* gene, on the other hand, which is stimulated by neuronal activity in the CNS [29–31], also is somewhat (~30%) activated in skeletal muscle by membrane depolarization.

To check if the myogenin gene, like genes coding for AChR subunits, is inhibited by PKC, the effect of the intramuscular administration of the phorbol ester PMA was examined. The results, shown in Fig. 2, resemble those obtained with electrostimulation except that they develop more slowly, perhaps as a consequence of the gradual penetration of the drug into the tissue, and also less completely, suggesting that the data reflect gene activities averaged over affected, less affected, and non-affected muscle fibers. At any rate, complete inactivation is not seen even after considerable time. Appropriate controls reveal that the effect is not caused by the vehicle or by some nonspecific phorbol ester action (Fig. 2).

The effect of blockade of PKC on the activity of myogenic factor genes was also investigated. We found that the membrane-to-genome signalling pathway was interrupted whether the enzyme was (a) acutely blocked

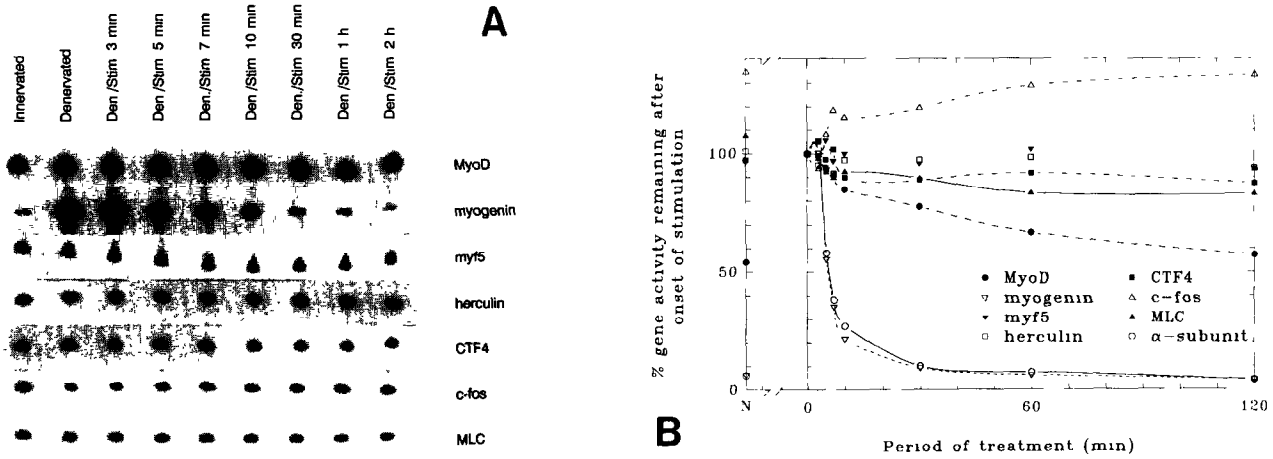

Fig. 1. Effect of electrostimulation on myogenic factor genes. Transcript elongation was assayed in nuclei isolated from control (innervated) muscle (N, 'normal') and from muscle denervated for 40 h and stimulated for the indicated periods of time. (A) Autoradiograms of ribonuclease protection analysis; the signals from the larger ones of the 2 myogenin and CTF4 protected regions (see section 2) are shown. (B) Quantitative analysis. Each data point represents the mean of up to 7 independent measurements; standard deviations averaged 5.4% (range: 0.1 to 8.1%). Results are normalized to the transcriptional activities in 40-h denervated muscle. About 1 h after the onset of stimulation, myogenin gene activity begins to fall below control (innervated muscle) values.

with the kinase inhibitor staurosporine or (b) depleted from the tissue by prior long-term exposure to phorbol esters (Fig. 3). As reported previously [25], the 3-day treatment with phorbol ester results in the virtually complete disappearance of PKC from skeletal muscle nuclei (data not shown).

The pharmacological experiments strongly suggest that PKC mediates the effects of electrical stimulation on the myogenin gene. Such a link implies that changes in gene activity should either accompany or follow, but not precede, changes in PKC activity. This prediction is borne out by observation: the rate of kinase activation is somewhat faster than the rate of gene inactivation; 5 min after the onset of stimulation nuclear PKC is about 60% activated (Fig. 4), while myogenin and α-subunit genes are blocked to a somewhat lesser extent (~45%) (Fig. 1). Since total (phorbol ester-induced) enzyme activity does not change, the increase in calcium/phosphatidyl serine-dependent protein phosphorylation is likely to be the consequence of activation of nuclear PKC rather than of translocation of cytosolic enzyme into the nucleus.

4. DISCUSSION

We have previously reported that the messages coding for the AChR α-subunit [32] and myogenin [23] are lost from electrically stimulated muscle with rates indistinguishable from those seen after actinomycin D administration, suggesting a sudden arrest of gene activity. The present report now confirms by direct gene activity analysis that the myogenin gene is indeed inactivated following plasma membrane depolarization. These findings and earlier observations concerning the presence and functional significance of E boxes in promoters of receptor subunits [8–17,20] and myogenin [33,34]; the

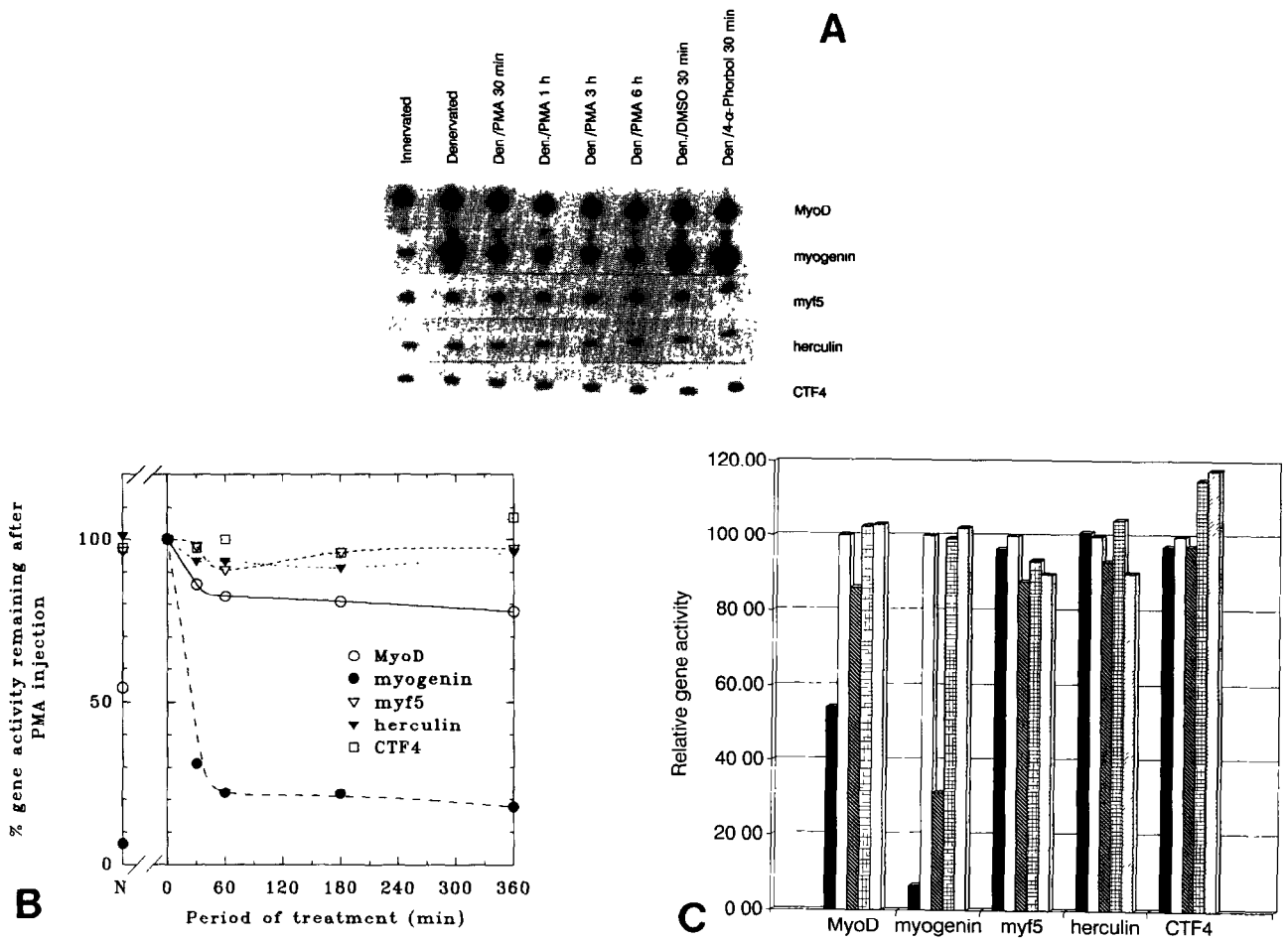

Fig. 2. Effect of phorbol ester on myogenic gene activity. Transcript elongation was assayed, as described in the legend to Fig. 1, in nuclei isolated from control (N) and denervated muscle, at the indicated times after in vivo administration of 20 μg phorbol 12-myristate 13-acetate (PMA)/μl 20% DMSO in ethanol (v/v), injected intramuscularly. (A) Autoradiograms of ribonuclease protection analysis. (B) Quantitative analysis of time course of PMA effect. Each data point represents the mean of 2 to 7 independent measurements; standard deviation averaged 3.4% (range 1.0 to 6.9%). (C) Quantitative analysis of pharmacological controls. The bar graph shows nuclear run-on data obtained with probes specific for the indicated genes. Within each set, results (from left to right) refer to: control (innervated) muscle; 40-h denervated; 40-h denervated/30-min PMA treated; 40-h denervated/30-min DMSO (20% DMSO in ethanol (v/v)) treated; and 40-h denervated/30-min 4-α-phorbol (Sigma; 20 μg in 20 μl of 20% DMSO in ethanol (v/v)) treated muscle. Results are normalized to the transcriptional activities in 40-h denervated muscle.

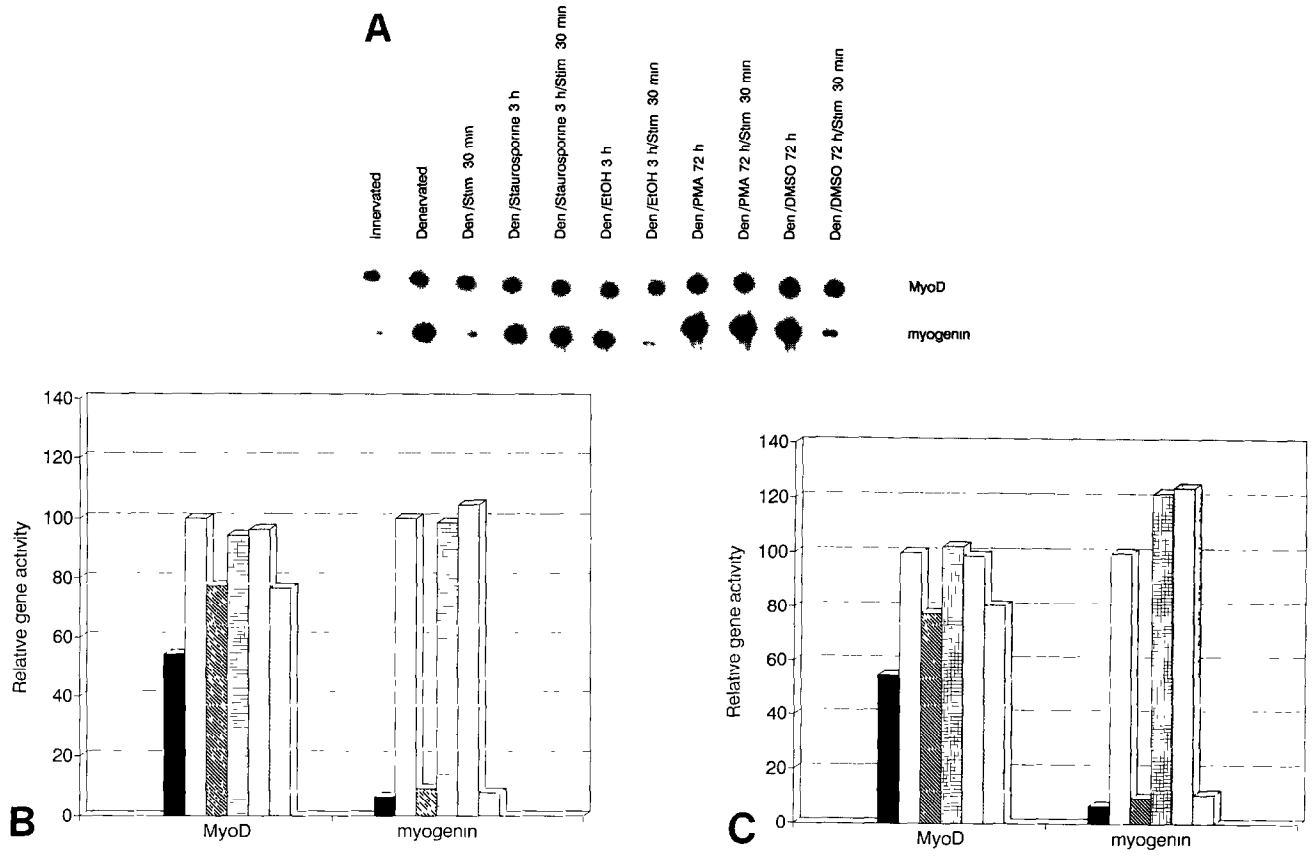

Fig. 3. Effect of blockade of PKC on MyoD and myogenin gene activities in stimulated muscle. At 37 h after denervation, 1 μg staurosporine (Sigma) in 20 μl 50% phosphate-buffered saline in ethanol (v/v), or ethanol alone, was injected into the denervated muscle. Three hours later, one group of animals received a 30-min stimulation while the control group remained unstimulated before being sacrificed. In a parallel experiment, animals received a 72-h treatment of PMA or DMSO alone. Similarly, one group received a 30-min stimulation while the control group remained unstimulated. Intramuscular injections of PMA and DMSO were repeated at 24-h intervals, with denervation taking place 32 h after administration of the first dose of PMA or DMSO (A) Autoradiograms of ribonuclease protection assay. (B) Quantitative analysis of staurosporine effect. The bar graph shows nuclear run-on data obtained with probes specific for MyoD and myogenin. Within each set, results (from left to right) refer to: control (innervated); 40-h denervated; 40-h denervated/30-min stimulated; 40-h denervated/3-h staurosporine treated; 40-h denervated/3-h staurosporine treated/30-min stimulated; and 40-h denervated/3-h ethanol treated/30-min stimulated muscle. Results of run-on assay are presented as % of gene activities in denervated muscle. (C) Quantitative analysis of PKC depletion effects. Transcription activities of MyoD and myogenin in nuclei isolated from (from left to right); control (innervated); 40-h denervated; 40-h denervated/30-min stimulated; 72-h PMA treated/40-h denervated; 72-h PMA treated/40-h denervated/30-min stimulated; and 72-h DMSO treated/40-h denervated/30-min stimulated muscle. The data are presented as % of gene activities in denervated muscle.

autocatalytic activity of MyoD and myogenin [35,36]; and kinetic evidence for an autocatalytic loop in denervation-induced receptor gene upregulation [23], impose constraints on models of the regulatory pathway. The simplest mechanism assumes that myogenin activates its own promoter. In this scheme, whose general outline was proposed by Changeux [37] and a myogenin version of which was sketched by Neville et al. [23], innervated muscle contains low levels of myogenin protein, which, as a result of nuclear PKC activity maintained by intermittent action potentials, is largely phosphorylated and consequently inactive. Denervation causes cessation of membrane activity; the resulting kinase inactivation and gradual dephosphorylation/activation of myogenin leads to the activation of myogenin-dependent genes, including the gene coding for myogenin itself. Conversely, when denervated muscle is stimulated, nuclear PKC is activated within minutes and through phosphorylation of myogenin protein shuts down myogenin-dependent genes including those coding for AChR subunits and for myogenin itself. That phosphorylation of myogenin by PKC indeed inactivates the factor has recently been reported [38]. Similarly, in cultured muscle cells activation of PKC rapidly leads to a decline of MyoD and myogenin mRNAs [39].

More complex mechanisms can be envisaged. For example, the feedback loop may contain an additional link or factor. That an indirect pathway for positive autoregulation of myogenin exists which involves the muscle-specific enhancer binding factor MEF-2, has been proposed by Olson and his collaborators [34,40]; the myogenin promoter in the chick as well as in mammals, in addition to a myogenin-sensitive E box also contains an MEF-2 site (TATATTT) which is crucial

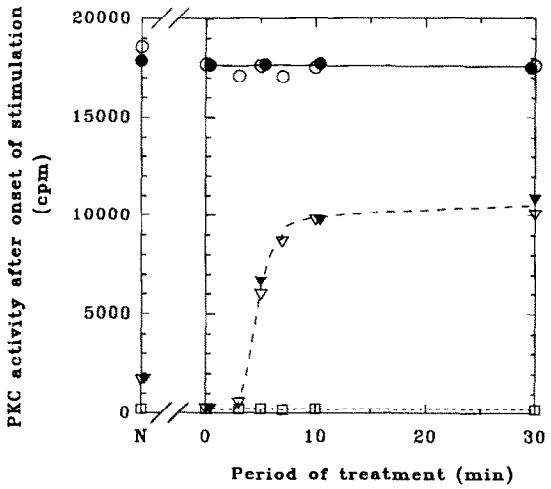

Fig. 4. Effect of electrostimulation on nuclear PKC activity. PKC enzyme activity was measured in nuclei from innervated muscle (N) and from muscle denervated for 40 h and stimulated for the indicated periods of time. Incubation mixtures contained, in addition to sample and buffer: calcium, phosphatidyl serine, and PMA (○, ●), calcium and phosphatidyl serine (△, ▲), and no addition (□); filled and open symbols refer to 2 independent experiments.

for muscle-specific activity [33,34]. It is conceivable therefore that MEF-2 is inactivated by the depolarization-PKC pathway, resulting in the shutting down of the myogenin gene and simultaneously of the AChR subunit genes. The latter effect, because of the absence of a measurable delay, is unlikely to be mediated by a depletion of myogenin protein; rather, a direct action of MEF-2 on receptor genes has to be postulated. So far MEF-2 sites have not been observed in receptor subunit promoters. The model nevertheless remains viable because neither the processes controlling the activity of the MEF-2 protein nor the regulatory regions of AChR subunit genes are known in their entirety. Myogenin may be subject to additional controls; thus protein kinase A also inhibits the activity of this factor [41]. In general, schemes in which an as yet unidentified common transcription factor plays a role or in which comparable kinetics in changes of gene activity result from the coincidence of parallel signalling pathways, although less likely, cannot be ruled out.

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