CaM Kinase II-dependent Suppression of Nicotinic Acetylcholine Receptor δ-Subunit Promoter Activity*

Received for publication, February 22, 2001, and in revised form, May 10, 2001
Published, JBC Papers in Press, May 11, 2001, DOI 10.1074/jbc.M101670200

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Nerve-induced muscle activity suppresses nicotinic acetylcholine receptor (nAChR) gene expression by increasing intracellular calcium levels. This suppression is mediated by nAChR promoter sequences harboring at least 1 E-box (CANNTG) that bind myogenic helix-loop-helix transcription factors. How muscle depolarization or increased calcium mediates changes in nAChR promoter activity is not well understood. In chick muscle, protein kinase C (PKC) activation is necessary for activity-dependent nAChR gene suppression. Similar effects of PKC activation have not been found in mammalian skeletal muscle. Therefore, we used rat primary muscle cultures to screen for other calcium-regulated enzymatic activities that may mediate the effects of muscle activity and calcium on nAChR promoter activity. We report here that calcium/calmodulin-dependent protein kinase II (CaM kinase II) can specifically suppress nAChR promoter activity in mammalian muscle. This regulation was mediated by a single E-box sequence residing in the previously characterized nAChR δ-subunit genes 47-base pair activity-dependent enhancer. In vitro protein/DNA interaction studies suggest that CaM kinase II inhibits binding of the myogenic factor, myogenin, to the δ-promoter 47-base pair activity-dependent enhancer. CaM kinase II activity is increased in active muscle and inhibition of this enzymatic activity results in increased nAChR δ-promoter activity. Therefore, CaM kinase II may represent a previously unappreciated activity that participates in coupling muscle depolarization to nAChR gene expression.

The nicotinic acetylcholine receptor (nAChR) is a pentameric integral membrane protein that mediates communication between nerve and muscle. During development of the neuromuscular junction the level, distribution, and properties of the nAChR change (1). Prior to muscle innervation or following muscle denervation, nAChRs are expressed at high levels throughout the muscles membrane and have a stoichiometry α3βγδ. After innervation these γ-containing receptors disappear and nAChRs with a stoichiometry α2βδ are found localized to the neuromuscular junction. The γ to ε-subunit switch during development results in expression of receptors with a shorter channel open time and higher conductance in adult, compared with embryonic, skeletal muscle (2).

The nerve plays an important role in the above patterns of nAChR expression. Nerve-derived neuregulins mediate selective expression of nAChR subunit encoding genes in end plate-associated nuclei, while nerve-induced muscle activity mediates suppression of nAChR gene expression in extrajunctional regions of the muscle fiber. Nerve-derived neuregulins mediate their effects via a Ras/mitogen-activated protein kinase signal transduction cascade that activates Ets family transcription factors and bind cis-acting regulatory sequences in the nAChR subunit gene promoters (3–5). Nerve-induced muscle activity suppresses nAChR gene expression via activation of a calcium-dependent signal transduction cascade (6, 7). In chick muscle, activity-dependent suppression of nAChR gene expression has been shown to require activation of protein kinase C (8, 9). Interestingly, there is little evidence for a PKC-dependent suppression of nAChR gene expression in mammalian muscle (10).

nAChR promoter elements necessary for conferring activity-dependent regulation have been identified in both birds and mammals (11–15). These elements contain one or more E-boxes (CANNTG), that bind myogenic basic helix-loop-helix transcription factors (12, 13, 16, 17). These factors are able to increase nAChR subunit promoter activity in transient co-transfection assays. Myogenin has emerged as the preferred helix-loop-helix myogenic factor mediating activity-dependent expression of nAChR subunit gene expression because: 1) its expression pattern is very similar to that of nAChR subunit genes (18–21); 2) it binds nAChR promoter activity-dependent enhancers (Ref. 22, and this report); and 3) it trans-activates nAChR promoters (17, 23, 24). In addition, myogenin DNA binding activity is suppressed by PKC activation (25). Therefore, it has been proposed that muscle activity suppresses nAChR subunit gene expression by a calcium-dependent activation of PKC that results in phosphorylation of myogenin, abrogating its ability to bind DNA (26).

Although the above mechanism of regulation is an attractive one, it is disconcerting that evidence for PKC-dependent regulation of nAChR gene expression in mammalian skeletal muscle is lacking (10). Previously we demonstrated that increasing intracellular calcium dramatically suppressed nAChR subunit gene expression in rat skeletal muscle (7, 10). However, the mechanism underlying this phenomenon is still not known.

In addition to being required for PKC activation, calcium also participates in activation of calcium/calmodulin-dependent protein kinases (CaM kinase) via its interaction with calmodulin. The CaM kinase family consists of CaM kinase I, II, and IV. CaM kinase II is encoded by four distinct genes, α, β, γ,
and δ (27). The kinase forms multimers of 8–12 subunits. CaM kinase II only requires calcium and calmodulin for activation. In contrast, CaM kinase I and IV are monomers and require phosphorylation by a CaM kinase prior to activation by calcium and calmodulin (28).

CaM kinases are multifunctional proteins, affecting numerous processes including gene expression, cell cycle progression, and synaptic plasticity (29). CaM kinases have been found in both cytoplasmic and nuclear cell fractions (27). This latter location suggests that CaM kinases may directly regulate transcription factor activity. Indeed, CaM kinase IV mediates calcium-dependent activation of CREB via phosphorylation of Ser133, while CaM kinase II inhibits CREB activity by phosphorylation of Ser142 (30). In skeletal muscle CaM kinase I and IV have been implicated in mediating calcium-dependent activation of MEP2 (31, 32) and the expression of muscle-specific genes.

Skeletal muscle cells express CaM kinase II genes encoding β-, γ-, and δ-subunits along with a truncated α-subunit (33–36). Although CaM kinase II can phosphorylate a number of substrates, a role in the regulation of skeletal muscle gene expression has not previously been reported. Here we report that CaM kinase II can repress nAChR promoter activity in muscle cells. This effect is specific to CaM kinase II, since a constitutively active version of CaM kinase IV had no effect. The cis-acting elements mediating the effect of CaM kinase II on nAChR δ-subunit promoter activity map to a previously identified 47-bp activity-dependent enhancer (15). An E-box sequence within this enhancer is necessary for CaM kinase II-dependent suppression. In vitro DNA binding assays showed that CaM kinase II activity impaired binding of a myogenin-containing protein complex to the nAChR δ-subunit promoter 47-bp activity-dependent enhancer, suggesting a mechanism for gene inactivation. These results identify CaM kinase II as a potent regulator of nAChR δ-subunit gene transcription and may provide a previously unappreciated mechanism for regulating these genes by calcium and muscle depolarization.

Materials and Methods

Cell Culture—Rat primary skeletal muscle cultures were prepared as previously described (37, 38). Cells were plated on collagen-coated 35-mm dishes at a density of $1 \times 10^5$ cells/ml and incubated at 37 °C under 8% CO₂. At 80% confluence, DNA transfection was performed. The C2C12 cell line was cultured in DMEM with 20% fetal bovine serum. At 90% confluence, the above medium was replaced with differentiation medium (DMEM, 2% horse serum) to induce cell differentiation. Six days later, myotubes were harvested for preparing nuclear extracts.

Plasmids—δ-550Luc contains a 550-bp promoter fragment of the nAChR δ-subunit gene driving luciferase expression (38). δ-47EMKLac contains the δ-subunit genes 47-bp activity-dependent enhancer upstream of the minimal enkephalin (MEK) promoter driving luciferase expression (15). δ-47Emut1MEKLac and δ-47Emut2MEKLac are the same as δ-47EMKLac, except the E box sequence has been mutated from CACCTG to CATATG in mut1 and to GCCCTG in mut2 (15).

Rat CaM kinase II γ-subunit cDNA was amplified from adult rat muscle by reverse transcription polymerase chain reaction with the upstream primer 1, CCGAGATTCGCCCGGTAGATGGCCACC, and the downstream primer 2, TGTCCTAGAGCCTAGCTCAGTCGAGCCTGCA. The cDNA was then cloned into the pcDNA2 vector between the EcoRI and XbaI sites. A constitutively active version of CaM kinase II γ-subunit was generated by restricting the full-length cDNA in pcDNA2 vector with NotI. This results in a 3' truncated product at codon 275 which removes the CaM kinase autoinhibitory and association domains. This constitutively active CaM kinase II γ-subunit encoding cDNA was subcloned into the NotI site of pcCS2MT. A kinase-dead version of CaM kinase II γ-subunit was generated by substituting Lys46 to Ala (K46A) using mutant primers GAGTAGTACGACGACATCTCATCAT and ATTGTAGATGGTCTGGCTGATACCT and the upstream and downstream primers 1 and 2 described above. Polymerase chain reaction products representing partial, but overlapping DNA sequence were annealed and the full-length K46A γ-subunit DNA was amplified using primers 1 and 2 described above. This mutant cDNA was cloned into the EcoRI/XhoI digested pcCS2MT and subsequently digested with Ncol to release a fragment identical to the constitutively active γ-subunit but harboring the K43A mutation. This K43A CaM kinase II γ-subunit fragment was subcloned into the Ncol site of pcCS2MT.

The full-length rat myogenin cDNA was amplified by polymerase chain reaction and then subcloned into pCS2MT vector. In the case of K43A CaM kinase II γ-subunit, the 5'-untranslated region (UTR) and XhoI site was amplified by polymerase chain reaction and subcloned into pCS2EI2 plasmid harboring the rat El2 cDNA under control of the simian CMV promoter. Both the pCMV CAT used for normalization, and the RSVLuc have been previously described (15). All constructs were confirmed by DNA sequencing.

DNA Transfection and Promoter Activity Assays—Primary muscle cell homogenates and then sera were digested with a 1.5 μg of DNA mixture (generally, 0.3 μg of reporter DNA, 0.2 μg of effecter DNA, 0.5 μg of normalizing DNA, and 0.5 μg of BSSK DNA) using Fugene-6 (Roche Molecular Biochemicals) according to manufacturer's directions. Twenty-four hours after transfection, the medium was changed to differentiating medium (5% horse serum, 10 μM Ringer's, and 2.8 μM/mL Ara-C). Three days later cells were harvested for luciferase and CAT assays as previously described (38, 40).

Nuclear Extracts—C2C12 cells were grown in DMEM containing 20% fetal calf serum and differentiated in DMEM containing 2% horse serum. Myotubes were harvested 6–9 days after differentiation. Nuclear extracts were prepared by the method of Dignam et al. (41) as modified by Amayr and Workman (42).

In Vitro Transcription/Translation—In vitro transcription/translation was performed according to the manufacturer's directions using a T7 SP6 Quick Coupled Transcription/Translation system (Promega). For each reaction, 40 μl of T7 Quick Master Mix, 1 μl of 1× methionine, and 2 μg of plasmid template were mixed together and the final volume adjusted to 50 μl with nuclease-free water. The mixture was incubated at 30 °C for 100 min.

DNA Binding Assay—Nuclear extracts (5–10 μg) or in vitro translated products were preincubated in 20 μl of binding buffer (10 μM HEPES (pH 7.9), 75 μM KCl, 1 mM EDTA, 1 mM diithiothreitol, 10% glycerol, 1.5 μg of poly(dI-dC)) either with or without anti-myogenin antibody (43, 45) antibody developed by Dr. Wright was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biology. Cells were treated with the constitutive active form of CaM kinase whose activity is independent of calcium/calmodulin and the specific substrate. These mixtures were incubated with reaction buffer containing 100 μCi/ml [γ-32P]ATP. The reaction was performed at 30 °C for 30 min and then stopped by ice. Mixes were then incubated with 32P-labeled DNA probe corresponding to the 47-bp activity-dependent enhancer from the nAChR δ-subunit promoter (15) for 45 min at reaction temperature. The reaction mixtures were incubated at 30 °C for 30 min and then stopped by reaction buffer containing 100 μCi/ml [γ-32P]ATP. The reaction was performed at 30 °C for 4 min, and then stopped by adding 15% ice-cold trichloroacetic acid. Reaction mixtures were centrifuged and the supernatant was spotted onto a phosphocellulose disc. Discs were washed with 1% H₃PO₄ buffer for 3 min, twice, and counted in a scintillation counter. The autonomous activity of CaM kinase II γ-subunit was then added to the mixture with 2 mM ATP, 0.3 mM PKC inhibitor, GF109203X, and 3 mM CaM-dependent protein kinase inhibitor peptide, PKI. These reaction mixtures were incubated at 30 °C for 30 min then stopped on ice. Mixes were then incubated with 32P-labeled DNA probe corresponding to the 47-bp activity-dependent enhancer from the nAChR δ-subunit promoter (15) for 30 min at room temperature. The DNA-protein complexes were resolved on high ion strength 5% polyacrylamide gels as described by Chodosh (44).

Calcium/Calmodulin-dependent Protein Kinase Activity Assay—Primary rat myotubes were grown in differentiation medium to examine the effects of muscle activity on CaM kinase activity, cells were washed with DMEM 3 times and incubated in differentiation medium ≥5 min. Cells were then either stimulated to contract using extracellular electrodes as previously described (15) or allowed to contract spontaneously. CaM kinase II activity was measured using a calcium/calmodulin-dependent protein kinase assay kit (Life Technologies, Inc.). Briefly, cells were lysed, pelleted, and supernatants were supplemented with either EGTA or EGTA and specific CaM kinase II substrate, or calcium/calmodulin and the specific substrate. These mixtures were incubated with reaction buffer containing 100 μCi/ml [γ-32P]ATP. The reaction was performed at 30 °C for 4 min, and then stopped by adding 15% ice-cold trichloroacetic acid. Reaction mixtures were centrifuged and the supernatant was spotted onto a phosphocellulose disc. Discs were washed with 1% H₃PO₄ buffer for 3 min, twice, and counted in a scintillation counter. The autonomous activity of CaM kinase II, i.e. calcium/calmodulin-independent activity, was calculated as percentage of the maximum CaM kinase activity. The assay has been repeated 3 times in individual experiments. Student t test was used to evaluate the significance of the results.

Results

CaM Kinase II-dependent Regulation of nAChR δ-subunit Promoter Activity—CaM kinase is composed of 3 domains: a N-terminal protein kinase domain, an inhibitory domain, and a C-terminal association domain (Fig. 1A). Removal of the inhibitory and association domains has been shown to result in a constitutive form of CaM kinase, whose activity is independent of calcium and calmodulin (45, 46). Constitutively active ver-
sions of CaM kinase were used in the following experiments to alleviate the need for upstream activating signals (see “Materials and Methods”).

A constitutively active version of CaM kinase II α-subunit (CaM kinase IIα) was previously shown to regulate the activity of transcription factors ATF-1 (47, 48) and CREB (30, 48). In addition, this activity also induces expression from prolactin and Rous sarcoma virus promoters (45). Therefore we first examined if an active CaM kinase IIα can regulate nAChRδ-promoter activity (Fig. 1B). Primary muscle cells were co-transfected with δ-550Luc and CMVCAT with or without active CaM kinase IIα, or active CaM kinase IV. Three days later cells were harvested for luciferase and CAT assays. Note a specific decrease in δ-promoter activity with CaM kinase IIα but not IV co-transfection. C, primary rat muscle cells were co-transfected with δ-550Luc and CMVCAT along with different amounts of active CaM kinase IIα. Three days later cells were harvested for luciferase and CAT assays. Experiments were done in quadruplicate and repeated at least twice. A dose-dependent suppression of δ-promoter activity was observed from 0.05 to 0.7 μg. Results are reported as a percent of basal δ-promoter activity that was set at 100%. Promoter activity is normalized luciferase activity. CMVCAT was used for normalization purposes.

For these experiments we cloned CaM kinase IIγ from rat skeletal muscle and created a constitutively active version similar to that of the α-subunit (30, 45). Transfection assays showed a dose-dependent decline in δ-promoter activity as increasing concentrations of active CaM kinase IIα were introduced into primary muscle cells (Fig. 1C). δ-Promoter suppression ranged from 5% at 0.05 μg to 86% at 0.7 μg of CaM kinase IIα DNA.

To confirm that the above effect of CaM kinase IIα on nAChRδ-promoter activity was the result of CaM kinase IIα enzymatic activity, we created a kinase-dead version of this enzyme. A mutation in the CaM kinase IIα ATP-binding site, that changed Lys43 to Ala (K43A) was generated. This mutation prevents ATP binding to CaM kinase and therefore renders it inactive (49, 50). Transfection of primary muscle cells with wild-type and K43A CaM kinase IIα showed that CaM kinase enzymatic activity is necessary for δ-promoter suppression (Fig. 2A).

CaM kinase IIα-dependent suppression of δ-promoter activity may represent a general effect on the basal transcriptional apparatus. In this case we would expect many promoters to respond in a similar fashion. Therefore, we examined the effect
active CaM kinase II had on RSV promoter activity in trans-
fected primary muscle cells. Unlike the pronounced suppres-
sion of nAChR-δ-subunit promoter activity (Fig. 2A), co-trans-
fectected active CaM kinase II, or the kinase-dead CaM kinase II (K43A). Only the catalyt-
ically active CaM kinase II was able to suppress δ-promoter activity. B, primary muscle cells were co-transfected with RSVLuc, CMVCAT, ± active CaM kinase II. Note that CaM kinase II had a slight activating effect on the RSV promoter. Transfections were done in quadruplicate and repeated at least twice. Promoter activity is normalized luciferase activity. CMVCAT was our normalization vector.

CaM Kinase II Activity Abrogates Myogenin-dependent Induction of the nAChR δ-promoter—Myogenin is known to trans-activate nAChR subunit promoter activity (17, 23, 24). This effect is mediated by E-box sequences within the nAChR subunit promoters (17, 22). Myogenin levels are high in cultured myotubes (51). Therefore, CaM kinase II may mediate δ-promoter suppression by acting on myogenin. One possibility is that active CaM kinase II suppressed myogenin expression resulting in reduced basal levels of δ-promoter activity in primary myotubes. If this were the case one would expect myoge-
in overexpression by a promoter not regulated by CaM kinase II to compensate for this hypothetical reduction in myogenin protein. Interestingly, CaM kinase II-dependent suppression of δ-promoter activity was not prevented by myogenin overexpression driven by the CMV promoter (Fig. 2A). This result sug-
gests that CaM kinase II may abrogate the ability of myogenin to bind and/or trans-activate the nAChR δ-promoter. Alterna-
tively, other δ-promoter-binding proteins may also participate in CaM kinase II-dependent suppression.

To determine if myogenin-dependent trans-activation is af-
fected by CaM kinase II activity, we employed an artificial promoter (4E-TK) harboring E-box sequences upstream of the minimal thymidine kinase promoter. NIH3T3 cells were trans-
fectected with 4E-TKLuc ± myogenin. We chose these cells so there would be no endogenous myogenin expression. Myogenin

**Fig. 2.** CaM kinase II catalytic activity differentially affects nAChR δ-promoter and RSV promoter activity. A, primary muscle cells were co-transfected with δ-550Luc, CMVCAT, ± active CaM kinase II, or the kinase-dead CaM kinase II (K43A). Only the catalytically active CaM kinase II was able to suppress δ-promoter activity. B, primary muscle cells were co-transfected with RSVLuc, CMVCAT, ± active CaM kinase II. Note that CaM kinase II had a slight activating effect on the RSV promoter. Transfections were done in quadruplicate and repeated at least twice. Promoter activity is normalized luciferase activity. CMVCAT was our normalization vector.

**Fig. 3.** Myogenin-dependent trans-activation of promoter ac-
tivity is abrogated by active CaM kinase II. A, primary muscle cells were co-transfected with δ-550Luc, CMVCAT, ± myogenin, active CaM kinase II, or kinase-dead (K43A) CaM kinase II. B, NIH 3T3 cells were co-transfected with 4E-TKLuc, CMVCAT, ± myogenin, and active CaM kinase II. Three days later cells were harvested for luciferase and CAT assays. Note that active, but not kinase-dead, CaM kinase II was able to inhibit myogenin-dependent induction of δ-promoter activity (A). In addition, active CaM kinase II also blocked myogenin-dependent induction of the 4E-TK promoter (B). Promoter activity is reported as lucif-
erase activity normalized to CAT activity. Experiments were done in quadruplicate and repeated at least twice.
overexpression in NIH3T3 cells resulted in about a 90% increase in promoter activity (Fig. 3B). However, when active CaM kinase II was included in the transfection, myogenin-dependent induction was abrogated (Fig. 3B), suggesting that CaM kinase II activity influences myogenin’s ability to bind and/or trans-activate this promoter.

CaM Kinase II-dependent Suppression of δ-Promoter Activity Is Mediated by the δ-Promoter 47-bp Activity-dependent Enhancer—We have previously shown that muscle activity and increased intracellular calcium mediate their effects on δ-promoter activity via a 47-bp enhancer sequence (7, 15). This sequence contains a single E-box that is necessary for activity and calcium-dependent gene expression (15). Here we examined if this enhancer can mediate the effects of CaM kinase II on nAChR δ-promoter activity. For these experiments we used a chimeric promoter harboring the δ-promoter 47-bp enhancer upstream of the MEK promoter driving luciferase expression (δ-47MEKLuc) (15). The MEK promoter was not regulated by active CaM kinase II (Fig. 4C).2 Transfection of muscle cells with δ-47MEKLuc, ± active CaM kinase II, showed that the 47-bp enhancer conferred CaM kinase II-dependent regulation onto the MEK promoter (Fig. 4A). In addition, myogenin was able to trans-activate this chimeric promoter and this activation was inhibited by active CaM kinase II (Fig. 4A). Therefore, the nAChR δ-subunit genes 47-bp activity-dependent enhancer confers CaM kinase II-dependent regulation onto a heterologous promoter.

We have previously demonstrated that the E-box sequence residing in the 47-bp activity-dependent enhancer was necessary for activity and calcium-dependent gene expression (7, 15). Here we examined whether this sequence is also necessary for CaM kinase II-dependent suppression. The 47-bp enhancer harboring E-box mutations, E-mut1 and E-mut2 were generated and ligated upstream of the MEK promoter (15) (Fig. 4B). Although E-box mutations decreased δ-47MEK promoter activity to less than 10%, this expression was still above background (Fig. 4C) and this residual promoter activity was not regulated by CaM kinase II overexpression (Fig. 4C).

CaM Kinase II Impairs Binding of a Myogenin Containing Complex to the δ-Promoter 47-bp Enhancer—The above results indicate that CaM kinase II activity can abrogate the ability of

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2 H. Tang and D. Goldman, unpublished data.
Regulation of nAChR Gene Expression

A.

\[
\begin{array}{c|cccccc}
\text{CaM Kinase II} & - & - & + & - & + & - \\
\text{CaM Kinase II (K43A)} & - & - & + & - & - & + \\
\text{Anti-Mgn Ab} & + & - & + & + & - & - \\
\end{array}
\]

Supershift →

\[
\begin{array}{ccc}
1 & 2 & 3 \\
\text{Probe} & \text{Lane 1} & \text{Lane 2} & \text{Lane 3} \\
\end{array}
\]

B.

\[
\begin{array}{cccccccc}
\text{Myogenin} & + & + & + & + & + \\
\text{E12} & + & + & + & + & - \\
\text{CaM Kinase II} & + & + & - & - & - \\
\text{Myogenin} & + & + & + & + & + \\
\end{array}
\]

\[
\begin{array}{cccccccc}
1 & 2 & 3 & 4 & 5 & 6 \\
\text{Probe} & \text{Lane 1} & \text{Lane 2} & \text{Lane 3} & \text{Lane 4} & \text{Lane 5} \\
\end{array}
\]

**Fig. 5.** *Active CaM kinase II inhibits binding of a myogenin-containing complex to the nAChR δ-subunit promoter 47-bp enhancer.* A, electrophoretic gel mobility shift assay showing binding of C2C12 nuclear extract to radiolabeled 47-bp enhancer. *Lane 1* shows binding of 2 complexes, labeled 1 and 2. *Lane 2* shows that inclusion of the anti-myogenin antibody causes a supershift of band 1, identifying band 1 as a myogenin-containing complex. *Lanes 3 and 4* compare the effect of active CaM kinase II on binding of this myogenin-containing complex. *Lanes 5 and 6* show that CaM kinase II enzymatic activity is necessary for reduced binding of the myogenin-containing complex. B, *in vitro* translated myogenin and E12 protein binding to the 47-bp enhancer is abrogated by active CaM kinase II. *Lanes 1–3* show that myogenin efficiently binds the enhancer as a myogenin:E12 heterodimer. *Lanes 4 and 5* show that this binding is inhibited by active CaM kinase II. *Lanes 6–9* show that CaM kinase has a larger effect on binding when first preincubated with myogenin or E12 prior to heterodimer formation. Labels (from top to bottom) above gels in B reflect the order of addition of components.

the δ-promoter 47-bp activity-dependent enhancer from activating gene expression in muscle cells. Reduced enhancer activity may result from decreased binding of trans-acting transcription factors to the 47-bp activity-dependent enhancer. Because myogenin-dependent trans-activation is abrogated by CaM kinase II activity it is possible that this enzyme inhibits myogenin binding to the 47-bp enhancer.

Myogenin binding to DNA was examined using electrophoretic gel mobility shift assays. Radiolabeled 47-bp activity-dependent enhancer was used as probe and the source of enhancer binding proteins was C2C12 myotube nuclear extract. *Fig. 5A, lane 1,* shows the result of mixing C2C12 nuclear extract with radiolabeled 47-bp enhancer DNA. Two retarded bands were detected on the gel (labeled 1 and 2). Competition with cold probe suggests that both these bands represent specific binding to the probe. To identify myogenin within these protein complexes, we included an anti-myogenin antibody in the binding reaction. This antibody caused a supershift of band 1 (*Fig. 5A, lane 2*), indicating this complex of proteins contains myogenin. Band 2 does not reproducibly change in intensity upon incubation with anti-myogenin antibody, suggesting this complex does not contain myogenin.

We next investigated if the binding of complex 1, containing myogenin, was reduced in the presence of active CaM kinase II (*Fig. 5A, lanes 3 and 4*). Indeed, *in vitro* synthesized active CaM kinase IIγ, when preincubated with C2C12 nuclear extract, decreased myogenin binding to the 47-bp enhancer (*Fig. 5A, lanes 3 and 4*). In contrast, an inactive kinase-dead version of CaM kinase II, (K43A) had little effect on myogenin binding (*Fig. 5A, compare lane 5 with lane 6*). These data suggest that the binding of a myogenin-containing complex to the 47-bp enhancer can be reduced by CaM kinase II enzymatic activity.

The δ-promoter 47-bp enhancer is comprised of three putative regulatory elements (an E-box sequence along with SV40 core enhancer and SP1-like-binding site sequences), each of which is necessary for enhancer activity (15). Therefore, CaM kinase II may result in reduced binding of a myogenin-containing complex to this enhancer via an effect on one or more of these binding proteins.

To investigate the effect CaM kinase II has on E-box binding proteins we used *in vitro* translated myogenin and E12, which bind to the E-box sequence as a complex (*Fig. 5B, lane 3*). Two major binding complexes are detected and labeled 1 and 2 in *Fig. 5B*. The faster migrating complex 2 is independent of myogenin and/or E12 expression and reflects an endogenous binding activity present in the *in vitro* translation mixture (*Fig. 5B, lanes 1 and 2*). In contrast, the slower migrating band, labeled 1, is dependent on expression of both myogenin and E12 (compare *lanes 1–3* in *Fig. 5B*). Consistent with our previous data, we found that preincubation of myogenin:E12 heterodimers with active CaM kinase IIγ reduced DNA binding (compare *lanes 4 and 5* in *Fig. 5B*).

CaM kinase II-mediated suppression of myogenin binding to the 47-bp enhancer may result, at least in part, by inhibiting myogenins interaction with E12. To address this issue, we first incubated myogenin or E12 with active CaM kinase IIγ. The reaction was then chilled on ice to inhibit CaM kinase II activity before adding myogenin or E12s binding partner (*Fig. 5B, lanes 6–9*). Interestingly, we found active CaM kinase IIγ caused a more dramatic suppression of binding under these conditions than when it was incubated with the myogenin:E12 complex (*Fig. 5B, compare *lanes 4 and 5* with 6 and 9*). Therefore, CaM kinase II may not only inhibit binding of myogenin: E12 heterodimers to their E-box sequence, but may also influence heterodimer formation. Both of these events would be expected to contribute to the observed CaM kinase II-dependent suppression of nAChR δ-promoter activity.

Absorption of CaM Kinase II Activity Increases nAChR δ-Promoter Activity in Contracting Myotubes—Although the above results suggest that CaM kinase II activity can suppress nAChR gene expression, they do not address whether this activity participates in suppression of nAChR gene expression in depolarized muscle. Therefore we first determined if CaM kinase activity increases upon depolarization of primary muscle myotubes. Previous experiments suggested that CaM kinase activity increases following stimulation of skeletal muscle with extracellular electrodes (53). However, we were using primary myotubes in our experiments and it was important to know if CaM kinase activity increased in these cells upon depolarization. Indeed, after 8 h of electrical stimulation, CaM
kinase increased ~70% over that assayed in inactive, tetrodotoxin-treated myotubes (Fig. 6). Similar results were obtained with spontaneously active muscle.

We next examined if inhibition of CaM kinase activity in active myotubes blocked activity-dependent suppression mediated by the nAChR δ-subunit promoter 47-bp activity-dependent enhancer. For these experiments we took advantage of the observation that our inactive CaM kinase IIδ (K43A) functions as a dominant-negative (Fig. 7A). Therefore, we transfected primary muscle cells with the δ-47MEKLuc reporter with and without the dominant-negative CaM kinase IIδ (K43A) (Fig. 7B). Microscopic observation confirmed that myotubes were spontaneously active. These experiments showed that blocking CaM kinase activity caused a 30% increase in reporter gene expression in active muscle, consistent with the idea that CaM kinase participates in activity-dependent gene suppression.

**DISCUSSION**

The main finding from this study is that CaM kinase II can suppress nAChR δ-subunit promoter activity by inhibiting binding of a myogenin-containing protein complex to the δ-promoter 47-bp enhancer. We had previously shown that this same enhancer mediates δ-promoter regulation by muscle depolarization and calcium (7, 15). Therefore, CaM kinase II is poised to play a role in mediating nAChR δ-subunit gene suppression in response to muscle activity and/or increased intracellular calcium.

Previous studies, in chick muscle, have suggested that muscle depolarization leads to decreased nAChR gene expression by activating a calcium-dependent PKC signal transduction pathway (8, 9). PKC was shown to be activated by muscle depolarization and this increased PKC activity was necessary for nAChR gene suppression. PKC can phosphorylate and block myogenin binding to its target E-box (25, 26) providing a mechanism for nAChR gene inactivation. Although this is an attractive mechanism, there is scant evidence for it mediating nAChR gene suppression in mammalian muscle (10). Because of this we searched for other signaling molecules that may participate in mediating the effects of muscle activity and increased intracellular calcium on nAChR gene expression.

CaM kinase was a logical choice for investigation since it is regulated by calcium and calmodulin. CaM kinases are ubiquitously expressed and have been reported to enter the nucleus to regulate gene expression via phosphorylation of specific transcription factors (27, 52). Most relevant to our studies is the observation that CaM kinase II δ is expressed in skeletal muscle (36) and CaM kinase II activity is enriched in myonuclei (52). In addition, muscle stimulation results in increased calcium, calmodulin, and CaM kinase activity (53).

We compared the effects of CaM kinase II with CaM kinase IV. Our experiments employed activated versions of these kinases that alleviated the need for treating cells with pharmacological agents that raise intracellular calcium. CaM kinase II is thought to have a broader substrate specificity than CaM
kinase II mediates d-sequences (Fig. 3). This effect required CaM kinase II enzymatic activity since a kinase-dead mutant, CaM kinase II (K43A), had no effect on δ-promoter activity (Fig. 2). CaM kinase II-dependent δ-promoter suppression does not result from a general effect on transcription since the RSV and CMV promoters were not suppressed by CaM kinase II (Fig. 2).²

Recently it was demonstrated that CaM kinase II activity is enriched in skeletal muscle nuclei and capable of phosphorylating serum response factor (52). Therefore, CaM kinase II may mediate its effect on nAChR δ-promoter activity by phosphorylation of specific nuclear transcription factors. One candidate for this regulation is the basic helix-loop-helix transcription factor myogenin. Myogenin activates nAChR δ-promoter activity and this activation is abrogated by active CaM kinase II (Fig. 3). The δ-promoter contains a number of different regulatory elements, each of which is a potential mediator of CaM kinase II effects. Therefore, to determine if CaM kinase II-dependent regulation can be mediated by E-box sequences we used a chimeric promoter containing 4 E-box sequences upstream of the minimal TK promoter in co-transfection assays with myogenin and CaM kinase II. Plasmids were transfected into NIH3T3 cells so the specific effect of myogenin could be examined. These experiments showed that active CaM kinase II can suppress myogenin-dependent trans-activation of an artificial promoter containing E-box cis-acting regulatory sequences (Fig. 3).

The above results are consistent with the idea that CaM kinase II mediates δ-promoter suppression by inhibiting myogenin activity to trans-activate this promoter. The δ-550 promoter used in the above experiments contains 5 E-boxes, each of which has the potential to mediate myogenin's effect (38). However, only one of these E-boxes is necessary for activity and calcium-dependent control of nAChR gene expression (7, 15). This latter E-box is part of a 47-bp enhancer that also contains elements similar to the SV40 core enhancer sequence and SP1 binding sequence (15). As expected CaM kinase II-dependent regulation mapped to this enhancer sequence (Fig. 4). These results are consistent with CaM kinase II participating in calcium and activity-dependent control of δ-promoter activity.

Is the 47-bp enhancer E-box sequence necessary for CaM kinase II-dependent regulation? Although our experiments using the 4E-box-TK promoter suggested that CaM kinase II can mediate its effects via an E-box binding sequence (Fig. 3), it was not clear if this sequence was necessary for CaM kinase II-dependent suppression of the 47-bp enhancers activity. We tested this by mutating the E-box in the 47-bp enhancer and examined its activity ± CaM kinase II (Fig. 4). Although the E-box mutation dramatically reduced promoter activity, it was still significantly above background levels. This allowed us to determine if CaM kinase II could suppress this activity further. Similar to our results using the 4E-box-TK promoter, we found that the 47-bp enhancer E-box is necessary for mediating CaM kinase II-dependent suppression (Fig. 4).

The above results suggested that CaM kinase II inhibited the activity or binding of myogenin or some other protein to the 47-bp enhancers E-box sequence. Therefore, we turned to electrophoretic gel mobility shift assays to investigate transcription factor binding to this sequence. We first documented that myogenin is present in muscle nuclear extracts and binds the 47-bp activity-dependent enhancer (Fig. 5A). These experiments showed a smear of binding activity within which 2 distinct bands can be observed. The slower migrating band contains myogenin as demonstrated by antibody mediated supershift (Fig. 5A). Interestingly, when active CaM kinase II was preincubated with the muscle cell nuclear extract there was a specific decrease in myogenin binding (Fig. 5A). This result suggests that CaM kinase II may directly regulate myogenin binding to the 47-bp enhancer. However, because muscle nuclear extracts were used in these experiments we could not rule out the possibility that CaM kinase targets another protein that binds the 47-bp enhancer in an E-box independent manner and indirectly influences myogenin binding.

To test if active CaM kinase II can inhibit myogenin binding to the 47-bp enhancer we used in vitro translated myogenin and its binding partner E12 in electrophoretic mobility shift assays. We first showed that myogenin does not readily bind to its target E-box without first forming a heterodimer with E12 (Fig. 5B, lanes 1–3). We then showed that this binding activity is reduced when heterodimers are preincubated with active CaM kinase II (Fig. 5B, lanes 4 and 5). However, when we repeated these experiments by first incubating in vitro translated myogenin or E12 with active CaM kinase II and then mixed myogenin and E12 together for binding, we found an even larger effect on binding (Fig. 5B, lanes 6–9). These results suggest that CaM kinase II may not only reduce binding of myogenin-E12 complexes to the 47-bp enhancer, but also may reduce their association with one another. Although these experiments highlight an effect of CaM kinase II activity on myogenin binding to the 47-bp enhancer, it is also possible that other proteins binding to this enhancer are also affected by this enzyme. However, characterization of the effect CaM kinase II has on these proteins must await their identification.

These results represent the first demonstration of a CaM kinase II-dependent signaling mechanism impinging on nAChR subunit gene expression. Other signaling systems implicated in regulating these genes include PKC, cAMP, c-Jun N-terminal kinase, and mitogen-activated protein kinase cascades. Of these cascades PKC, c-Jun N-terminal kinase, and cAMP systems seem to participate in activity-dependent gene expression (6, 9, 38, 54), while mitogen-activated protein kinase and c-Jun N-terminal kinase signaling appear to contribute to synapse-specific expression of these genes (55–58). Our results suggest that CaM kinase II signaling can be added to the list of transduction cascades that regulate nAChR gene expression. In addition, we showed that CaM kinase activity is increased in active versus inactive myotubes (Fig. 7) and that abrogation of this increased activity with a dominant-negative version of CaM kinase resulted in increased activity from the δ-promoter 47-bp activity-dependent enhancer. Therefore, CaM kinase activity appears to participate in activity-dependent suppression of δ-promoter activity in primary rat myotubes.

Whether one of the above listed signaling cascades predominates in any particular species (for example, PKC-dependent signaling may predominate in chick but not rat) or has a larger effect on controlling expression of a subset of nAChR subunit genes is not yet clear and needs to be further investigated. Nonetheless, it is clear from the studies reported here that CaM kinase II can regulate rat muscle nAChR δ-promoter activity via myogenin binding and therefore has the potential to regulate other subunit genes which also contain E-boxes that mediate the effects of muscle activity and calcium.

Acknowledgments—We thank Dr. D. L. Turner for the pCS2E12 plasmid, Drs. Howard Schulman and Richard Maurer for sharing CaMKII and IV cDNAs, and Dr. M. Uhler for advice on mutagenesis. We also thank members of the Goldman lab for helpful discussions.

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