Identification of an Element Required for Acetylcholine Receptor-inducing Activity (ARIA)-induced Expression of the Acetylcholine Receptor \( \epsilon \) Subunit Gene

(Received for publication, January 21, 1997, and in revised form, February 25, 1997)

Jutong Si, Daniel S. Miller, and Lin Mei‡

From the Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Acetylcholine Receptor (AChR)-inducing activity (ARIA) is believed to be the trophic factor utilized by motoneurons to stimulate AChR synthesis in the subsynaptic region of the muscle. Among the four AChR subunit genes, the \( \epsilon \) subunit gene is strictly expressed in nuclei localized to the synaptic region of the muscle. To understand mechanisms of the regulation of synapse-specific transcription, we studied the promoter activity of the 5'-flanking region of the AChR \( \epsilon \) subunit gene in response to ARIA. Transgenes containing the wild type or mutant 5'-flanking regions upstream of a luciferase gene were transfected in C2C12 muscle cells. The promoter activity of these transgenes was determined by assaying activity of expressed luciferase. Analyzing a combination of 5' deletion and site-directed mutants, we identified a 10-nucleotide element (position \(-55/46\)) which was crucial for ARIA-induced expression from the \( \epsilon \) subunit promoter. This element was named ARE for ARIA-responsive element. Mutation of ARE greatly diminished ARIA-induced transgene expression and deletion of ARE abolished completely the ARIA response. Electrophoretic mobility shift analyses revealed a DNA binding activity in muscle nuclear extract that interacted with ARE. Such interaction was enhanced by ARIA stimulation of muscle cells and appeared to be dependent on nuclear protein phosphorylation.

The development and maintenance of a functional neuromuscular junction require that expression of all the molecular components be temporally and spatially regulated at the nerve-muscle contact. For example, the acetylcholine receptor (AChR) is highly enriched at the crests of the postjunctional muscle contact. For example, the acetylcholine receptor subunit gene is strictly expressed in nuclei localized to the synaptic region of the muscle. To understand mechanisms of the regulation of synapse-specific transcription, we studied the promoter activity of the 5'-flanking region of the AChR \( \epsilon \) subunit gene in response to ARIA. Transgenes containing the wild type or mutant 5'-flanking regions upstream of a luciferase gene were transfected in C2C12 muscle cells. The promoter activity of these transgenes was determined by assaying activity of expressed luciferase. Analyzing a combination of 5' deletion and site-directed mutants, we identified a 10-nucleotide element (position \(-55/46\)), which was crucial for ARIA-induced expression from the \( \epsilon \) subunit promoter. This element was named ARE for ARIA-responsive element. Mutation of ARE greatly diminished ARIA-induced transgene expression and deletion of ARE abolished completely the ARIA response. Electrophoretic mobility shift analyses revealed a DNA binding activity in muscle nuclear extract that interacted with ARE. Such interaction was enhanced by ARIA stimulation of muscle cells and appeared to be dependent on nuclear protein phosphorylation.

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Experimental Procedures

Materials—The recombinant ARIA (rHRG\(_{177-244}\)), a peptide of HRG\(_{177-244}\) was generously provided by Dr. Mark Slwikowski (21). Calf intestinal phosphatase was purchased from Boehringer Mannheim. All other chemicals, including okadaic acid, were purchased from Sigma.

Transgene Constructs—Deletion mutants of the \( \epsilon \) subunit promoter were generated by polymerase chain reaction (PCR) using the p5300-\( \Delta L \), plasmid as a template. The upstream primers with a HindIII site were 5'-GGG AGA AGC TTC TCT CCT GCA GAG CAC for primer (1) for \(-416\)-Luc, 5'-GGG AGA AGC TTC TCT CCT GCA GAG ACA GG for \(-170\)-Luc, 5'-GGG AGA AGC TTC GGG CAG CTT CCA GCC for \(-78\)-Luc, and 5'-GGG AGA AGC TTC CCA CCA GAG CAT GAT for \(-45\)-Luc. The downstream primer (Primer 2) was 5'-GGG AGA AGC TTC AGG GAA

*This work was supported in part by a National Institutes of Health Grant NS34062. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Virginia School of Medicine, Box 448, Jordan Hall 515, 1300 Jefferson Park Ave., Charlottesville, VA 22908. Tel.: 804-982-4440; Fax: 804-982-3878; E-mail: lm5u@virginia.edu.
CAG G with a HindIII site. Point and internal deletion mutants of the $\epsilon$ subunit promoter were generated by the method of PCR overlap extension (22). The pE3500-nlacZ plasmid was used as a template in the first PCR reaction using Vent DNA polymerase (New England Biolabs). The sense oligonucleotides of the intended overlapping primers (with mutated bases underlined) were 5'-GGG ACA ACT TGG GGG GCA GCT G (position –89/-68) for the ets mutant, 5'-ATG GGG CAG CTT CCT CAT AAA CCC CCA CAG CAG GGG C (position –79/-43) for the AP-2 mutant, 5'-CTG CCT CCC CCA CCC AAG CAG CTT TGG CAG ACG ATT AGG TGA (position –70/-28) for the ARE mutant, 5'-CTG CCT CCC CCA CCC GGC AGA GGA TTA GGT GA (position –70/-28) for the ARE deletion mutant, and 5'-GCT GAC AGT CCG CCC CAA ACC TAG CTA TCT CAC ACC TCC TTC ACA (position –33/-18) for the N-box mutant. The two overlapping DNA fragments from the first PCR reaction were used as templates in the second PCR reaction with Primers 1 and 2. After digestion with HindIII, the PCR products were subcloned in pGLOBasic (Promega) upstream of the luciferase gene. The authenticity and orientation of the synthetic promoter fragments were verified by DNA sequencing.

Cell Culture, Transfection Procedures, and Luciferase and $\beta$-Galactosidase Assays—C2C12 cells were cultured as described previously (17). C2C12 myoblasts at approximately 50% confluence were co-transfected with an $\epsilon$ subunit promoter-luciferase transgene (20 $\mu$g of DNA) and a control plasmid (1 $\mu$g of DNA) (pCMVb), which encodes $\beta$-galactosidase, by the standard calcium phosphate technique (23). Luciferase assay was performed using a kit from Promega following the manufacturer's instruction. $\beta$-Galactosidase activity was determined as described previously (17). Luciferase activity of transgenes was normalized to $\beta$-galactosidase activity to correct for variations in transfection efficiency.

Electrophoretic Mobility Shift Assays—C2C12 cells were washed in phosphate-buffered saline and homogenized in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Cell nuclei were pelleted by centrifugation at 4,000 rpm for 15 min at 4 °C. The pellets were extracted in DNA binding buffer containing 12% glycerol, 12 mM HEPES-NaOH, pH 7.9, 4 mM Tris/HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml pepstatin, 1 $\mu$g/ml leupeptin, and 2 $\mu$g/ml aprotinin. Nuclear extract protein (10 $\mu$g) was incubated with 2 $\mu$g of nonspecific competitor poly(dIdC), 4.5 $\mu$g of bovine serum albumin, and 1 ng of $^{32}$P-labeled double-stranded oligonucleotide probe (~5,000 cpm) in a final volume of 15 $\mu$L of DNA binding buffer. After incubation at 30 °C for 15 min, the reaction was stopped by the addition of 5 $\mu$L of loading buffer and run onto a 4% acrylamide gel (24). The radiolabeled probes were 5'-CCC CAC AGC AGG GG for ARE or 5'-CTA GCC CCG AAC TAA for N-box (25). In some experiments, unlabeled double-stranded oligonucleotides were used as a competitor, including the ARE or N-box wild type, ARE mutant (5'-CCA AGC AGC GTT GG), or SRE wild type (5'-CCC CAT ATT AGG GG).

RESULTS AND DISCUSSION

Localization of the ARIA-responsive Promoter Activity in 34 Nucleotides between –78 and –45 in the $\epsilon$-Flanking Region of the $\epsilon$ Subunit Gene—To determine the minimum length of the 5′ regulatory region required for promoter activity in response to ARIA, we generated transgenes containing a series of deletion mutants in the 5′-flanking region of the $\epsilon$ subunit gene. Transgenes were transfected in C2C12 muscle cells, and promoter activity was characterized by luciferase assay in ARIA-stimulated C2C12 myotubes. Expression of the –416-Luc transgene, which contains 416 nucleotides upstream from the transcription initiation site, was increased by ARIA stimulation in a concentration-dependent manner (Fig. 1). The maximal response (around 2-fold) was achieved with 1 nM ARIA, which agrees well with our previous observation using pE3500nlacZ in which expression of $\beta$-galactosidase is driven by the 3.5-kilobase 5′-flanking sequence of the $\epsilon$ subunit gene (17). These results suggest that the promoter element(s) required for the ARIA response is contained within 416 nucleotides upstream of the transcription initiation site. Therefore, the –416-Luc transgene was considered as equivalent to wild type for the purposes of this study. The ARIA response was specific to the $\epsilon$ subunit promoter, since the promoter of rat skeletal muscle voltage-sensitive sodium channel subtype 2 (skm-2) did not respond to ARIA stimulation (Fig. 1C). Promoter activities of the transgene deletion constructs –170-Luc, –78-Luc, and –45-Luc in myotubes in response to 1 nM ARIA are shown in

![Fig. 1. Requirement of 34 nucleotides (position –78/–45) in the $\epsilon$ subunit promoter for ARIA-stimulated $\epsilon$ subunit gene expression. A, schematic diagram of wild type (–416-Luc) and deletion mutants of the $\epsilon$ subunit promoter-luciferase transgene. Arrows indicate the transcription initiation site. B, concentration-dependent stimulation of the –416-Luc transgene expression by ARIA. C, relative luciferase/$\beta$-galactosidase activity of wild type (–416-Luc) and deletion mutants. Transgenes were co-transfected with pCMVb in C2C12 myoblasts. The promoter of rat skeletal muscle voltage-sensitive sodium channel subtype 2 (skm-2) was used as a control. D, concentration-dependent stimulation of the –45-Luc transgene expression by ARIA. After formation of myotubes, cells were stimulated without or with 1 nM ARIA (unless otherwise specified) for 24 h. Equal amounts of cell lysates were assayed for luciferase and $\beta$-galactosidase activity. These data represent the mean ± S.D. of three or more independent experiments. Open histograms indicate basal relative luciferase/$\beta$-galactosidase activity, i.e. in the absence of ARIA. Hatched histograms indicate ARIA-stimulated relative luciferase/$\beta$-galactosidase activity. *, $p < 0.05$; **, $p < 0.01$, in comparison with basal.
Identification of a 10-Nucleotide Element (ARE) Required for ARIA-stimulated \(\epsilon\) subunit gene expression. A, nucleotide sequence of the 5'-flanking region of the mouse AChR \(\epsilon\) subunit gene. The sequence of 416 nucleotides upstream of the transcription initiation site (+1) is presented. Consensus sequences for CARG, E-box, AP-2, and N-box are indicated. The ets and ARE cis-elements are underlined with solid lines. B, comparison of wild type and mutant sequences of ets, AP-2, ARE, and N-box. A hyphen indicates no change in sequence. All mutants \((m)\) were made in the −416-Luc construct, which is considered as equivalent to wild type (WT). C, relative luciferase/β-galactosidase activity of site-directed mutant transgenes. Luciferase activity was assayed in transfected myotubes and normalized to β-galactosidase activity. Open histograms indicate basal relative luciferase/β-galactosidase activity, i.e., in the absence of ARIA. Hatched histograms indicate ARIA (1 nM)-stimulated relative luciferase/β-galactosidase activity. These data represent the mean ± S.D. of at least three independent experiments. **, \(p < 0.01\) in comparison with basal.

A.

**Fig. 1C.** Seventy-eight nucleotides upstream from the transcription initiation site of the \(\epsilon\) subunit gene were sufficient to confer an ARIA response. This is in agreement with previous observations that 150 and 83 nucleotides upstream of the transcription initiation site of the \(\epsilon\) subunit gene are sufficient to respond to ARIA (15) and to confer preferential synaptic expression (25), respectively. The transgene containing 45 nucleotides, however, failed to respond to ARIA, even at concentrations up to 10-fold higher than required for a maximal response with the wild type transgene (Fig. 1D). The difference in the promoter activities between −78-Luc and −45-Luc indicated that the element required for ARIA response could be localized in the 34 nucleotides between −78 and −45.

**Identification of a 10-Nucleotide Element (ARE) Required for ARIA-induced \(\epsilon\) Subunit Expression**—We previously demonstrated that ARIA-stimulated AChR gene expression requires MAP kinase activation (17). Major transcription factors mediating MAP kinase action in mammalian cells include ternary complex factor proteins (for the ets element) (26) and SRF (for the SRE element) (27). Anticipating that the ARE-responsive element might be localized between −78 and −45, we identified and selectively mutated a number of sites in this region, concentrating on elements that could potentially bind transcription factors regulated by the MAP kinase pathway. Among the sites mutated, a ten-nucleotide element (CCA CAG CAG G, position −55/−46) was included. This element was chosen because it resembles SRE, having two cytidines on the 5' end and two guanosines on the 3' end (27). However, it differs from SRE in that the intervening 6 nucleotides have two cytidines and one guanosine instead of purely adenosines or thymidines as in the consensus SRE. Replacement of adenosines or thymidines with cytidines or guanosines in the internal 6 nucleotides dramatically decreases or diminishes SRE binding activity (28). In addition, the putative ets site (CAG GAT), which we identified at −83/−78, the putative AP-2 element (position −64/−54), and the N-box element (−11/−6), which was recently found to be required for synapse-specific expression (28), were also mutated. All mutants were made in the −416-Luc construct. While ets and AP-2 mutants diminished basal expression, they had no effect on ARIA-stimulated expression. By contrast, mutation of the 10-nucleotide SRE-like element attenuated greatly or abolished the \(\epsilon\) promoter activity in response to ARIA (Fig. 2). Myotubes possessing this mutant transgene failed to respond to concentrations of ARIA sufficient to activate the wild type transgene (Fig. 3A). However, an increase in mutant transgene expression was observed at 10 nM ARIA. These data strongly suggested that this 10-nucleotide element may be required for the ARIA-induced \(\epsilon\) subunit gene expression. We named this element ARE for ARIA-responsive element. To confirm that ARE is required for the ARIA response, we tested the promoter activity of an ARE deletion mutant (ARE.Δ). As shown in Fig. 3B, the ARE.Δ mutant promoter failed to confer ARIA response even at 10 nM of ARIA. While this work was in progress, Ducrè et al. (25) reported that the N-box is crucial for synaptic

**Fig. 2.** Requirement of ARE for ARIA-stimulated \(\epsilon\) subunit gene expression. A, nucleotide sequence of the 5'-flanking region of the mouse AChR \(\epsilon\) subunit gene. The sequence of 416 nucleotides upstream of the transcription initiation site (+1) is presented. Consensus sequences for CARG, E-box, AP-2, and N-box are indicated. The ets and ARE cis-elements are underlined with solid lines. B, comparison of wild type and mutant sequences of ets, AP-2, ARE, and N-box. A hyphen indicates no change in sequence. All mutants (\(m\)) were made in the −416-Luc construct, which is considered as equivalent to wild type (WT). C, relative luciferase/β-galactosidase activity of site-directed mutant transgenes. Luciferase activity was assayed in transfected myotubes and normalized to β-galactosidase activity. Open histograms indicate basal relative luciferase/β-galactosidase activity, i.e., in the absence of ARIA. Hatched histograms indicate ARIA (1 nM)-stimulated relative luciferase/β-galactosidase activity. These data represent the mean ± S.D. of at least three independent experiments. **, \(p < 0.01\) in comparison with basal.

**Fig. 3.** Response of ARE-mutated transgenes to ARIA stimulation. A, response of ARE point mutant transgene (ARE.m) to indicated concentration of ARIA. B, response of ARE-deletion mutant transgene (ARE.Δ) to indicated concentration of ARIA. The mutant transgenes were co-transfected with pCMVβ in C2C12 myoblasts. After ARIA treatment, C2C12 myotubes were lysed and assayed for luciferase and β-galactosidase activity. The presented luciferase activity was normalized to β-galactosidase activity. Open histograms indicate the basal activity, whereas hatched histograms indicate the promoter activity in response to ARIA stimulation. These data represent the mean ± S.D. of three or more independent experiments. **, \(p < 0.01\) in comparison with basal.
expression of AChR $\varepsilon$ subunit gene. Using an in vivo expression assay in intact muscles, the authors observed that 5' deletion mutants (-83 and -75) of $\varepsilon$ subunit promoter provided approximately the same level of expression, whereas -63 and -52 5' deletion mutants gave a decreased expression and the -36 nucleotide construct did not express. This observation prompted that authors to suggest "the presence of an activating element" in this region. In addition, site-directed mutation in this region diminished synaptic expression of the $\varepsilon$ subunit gene (25). We believe that ARE may be the activating element in this region crucial for synaptic expression.

Interaction between ARE and a Nuclear Protein Was Enhanced by ARIA Stimulation of Muscle Cells and Dependent on Phosphorylation of Nuclear Protein—Electrophoretic mobility shift analyses were performed to determine if ARE was able to interact with nuclear proteins in ARIA-stimulated myotubes. Using [$\gamma$-$^{32}$P]ATP-labeled double-stranded oligonucleotides containing the ARE element as a probe, we detected a prominent complex on the autoradiogram of a 4% acrylamide gel (Fig. 4A). The interaction between the ARE probe and nuclear binding protein was specific. First, formation of the complex was competitively inhibited by a 10- or 100-fold excess of unlabeled double-stranded wild type N-box oligonucleotides. In contrast, ARE mutant oligonucleotides required a much higher concentration to disrupt the complex formation. Second, 100-fold excess of unlabeled double-stranded wild type are oligonucleotides was almost completely inhibited by the specific ARE probe (Fig. 4A), suggesting that ARE interacted with a protein different from those that interact with SRE. Interestingly, formation of the specific complex was enhanced by ARIA stimulation of C2C12 myotubes. Treatment of myotubes with ARIA increased the binding activity by at least 2-fold (Fig. 4B). These results suggested that ARE interacted with a nuclear protein(s).

Knowing that activation of MAP kinase is required for ARIA-induced AChR gene expression (17, 18), we attempted to determine whether the ARE nuclear binding activity was regulated by MAP kinase activity. Myotubes were treated with PD98059, an inhibitor of MAP kinase kinase, which is able to disrupt MAP kinase activation and ARIA-induced AChR gene expression in C2C12 cells (17). The ARE binding activity was decreased in PD98059-treated myotube nuclear extract (Fig. 4B). Incubation of myotube nuclear extract with calf intestinal phosphatase to dephosphorylate nuclear proteins attenuated

**FIG. 4.** Characterization of the ARE binding activity using electrophoretic mobility shift assays. A, specificity of the ARE nuclear binding activity. ARIA (1 nM)-stimulated nuclear extract was used for electrophoretic mobility shift assays. The reaction was incubated in the absence (CONTROL) or presence of unlabeled N-box oligonucleotides. B, enhancement of the ARE nuclear binding activity by ARIA stimulation. 25C12 myotubes were treated without or with 1 nM ARIA in the absence or presence of 20 nM PD98059. As a control, bovine serum albumin (BSA) was used instead of nuclear extract (Nuc. Extr.) in the reaction shown on the left lane. Nuclear extract was isolated and incubated with a [$\gamma$-$^{32}$P]ATP-labeled DNA probe containing the ARE element. DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel. The prominent specific binding complex is indicated by an arrow. C, dependence of ARE binding activity on phosphorylation of nuclear proteins. Prior to the reaction, nuclear extract from ARIA (1 nM)-treated myotubes was incubated without or with indicated concentrations of calf intestinal phosphatase (CIP). The incubation condition was as follows: CONTROL, 4°C for 15 min alone; CIP, 30°C for 15 min in the presence of calf intestinal phosphatase; PRE-INC, 30°C for 15 min alone; PRE-INC + 2 nM OA, 30°C for 15 min in the presence of 2 nM okadaic acid. The prominent specific binding complex is indicated by an arrow.

**FIG. 5.** Characterization of the N-box binding activity using electrophoretic mobility shift assays. A, specificity of the N-box nuclear binding activity. ARIA (1 nM)-stimulated nuclear extract was used for electrophoretic mobility shift assays. The reaction was incubated in the absence (CONTROL) or presence of unlabeled wild type N-box oligonucleotides. B, independence of the N-box nuclear binding activity on ARIA stimulation. 25C12 myotubes were treated with or without 1 nM ARIA in the absence or presence of 20 nM PD98059. Nuclear extract was isolated and used for electrophoretic mobility shift assays with a [$\gamma$-$^{32}$P]ATP-labeled double-stranded probe containing the N-box element. The prominent specific binding complex is indicated by arrows.
the ARE binding activity in a concentration-dependent manner (Fig. 4C). There appeared to be a high level of endogenous phosphatase activity in myotube nuclear extract. Preincubation of myotube nuclear extract at 30 °C for 15 min alone, without the addition of exogenous phosphatase, was able to decrease ARE binding activity (Fig. 4C). Moreover, the preincubation-induced decrease in ARE nuclear binding activity was blocked by 2 nM okadaic acid, a specific inhibitor of serine/threonine phosphatases (Fig. 4C). Thus the ARE binding activity may derive from a phosphoprotein that has an enhanced DNA binding activity in the phosphorylated state. The phosphatase activity in C2C12 myotubes may be due to protein phosphatase 2A, a phosphatase which is specifically inhibited by okadaic acid at a nanomolar concentration (29).

N-box Is Not Required for ARIA-stimulated £ Subunit Gene Expression in C2C12 Cells—Recently, a cis-element of 6 nucleotides (−11/−6) in the £ subunit promoter was identified and implicated in compartmentalized expression of AChR genes at the neuromuscular junction (25). We found that although mutating the N-box dramatically decreased basal expression of the transgene, the N-box mutant promoter was still able to respond to ARIA stimulation (Fig. 2C). Using the N-box probe, we detected a binding activity in the C2C12 myotube nuclear extract (Fig. 5A), confirming the previous observation (25). This binding activity was specific, since it was inhibited by excess unlabeled double-stranded N-box oligonucleotides (Fig. 5A). Stimulation of C2C12 cells with ARIA, or with ARIA in the presence of PD98059, did not appear to affect the N-box binding activity (Fig. 5B). These results suggest that N-box may not be crucial for ARIA-induced £ subunit gene expression in C2C12 cells.

In summary, we have identified ARE, a 10-nucleotide element (CCA CAG CAG G, position −55/−46) in the £ subunit promoter, which was required for ARIA-induced expression of £ subunit gene in C2C12 muscle cells. ARE was able to interact with a nuclear protein(s). Furthermore, interaction between ARE and the nuclear protein was enhanced by ARIA stimulation of muscle cells and seemed to depend on phosphorylation of nuclear proteins. Experiments to elucidate the identity of the ARE binding protein are currently in progress.

Acknowledgments—We thank Dr. Wen C. Xiong for advice; Drs. David Brautigan, Doug Bayliss, and Sheridan Swope for critical comments; and Sandra Won, Michael Tanowitz, and Dr. Yi Sun for discussion. Our sincere appreciation goes to Dr. Gerry Chu and Dr. Joshua Sanae for pE5500-nlacZ DNA; Dr. Evelyn Ralston for C2C12 cells; Dr. Alan Saltiel for PD98059; Dr. Mark Sliwkowski for recombinant ARIA; and Roland Kallen for the skm-2 transgene.

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J. Biol. Chem. 1997, 272:10367-10371.
doi: 10.1074/jbc.272.16.10367

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