Phosphorylation of Myogenin in Chick Myotubes: Regulation by Electrical Activity and by Protein Kinase C. Implications for Acetylcholine Receptor Gene Expression†

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ABSTRACT: We have analyzed the potential role of myogenin in the regulation by electrical activity of the expression of the acetylcholine receptor (AChR) α-subunit gene in cultured chick embryonic myotubes. The state of phosphorylation of myogenin was followed by ^{32}P-labeling and immunoprecipitation with an anti-myogenin antibody. In electrically active myotubes myogenin is phosphorylated, while it is dephosphorylated in electrically silent myotubes following tetrodotoxin (TTX) treatment. Accordingly, nuclear protein kinase C (PKC) activity decreases in TTX-treated myotubes. Myogenin dephosphorylation is also observed upon incubation of myotubes with GF109203X, a pharmacological agent which specifically inhibits PKC activity. Both treatments cause similar increases in the expression of the AChR protein. The effects are not additive. Thus TTX and GF109203X most probably affect a common process. Recombinant chick myogenin binds to myogenic sites (E boxes) present in the AChR α-subunit promoter but loses this binding capacity after phosphorylation. As a working hypothesis we propose that repression of AChR biosynthesis by electrical activity results, at least partly, from phosphorylation of myogenin via the PKC pathway.

In adult skeletal muscle, the distribution of the acetylcholine receptor protein (AChR)[1] is restricted to the junctional area [reviewed in Salpeter and Lohring (1985)]. Outside the endplate, muscle electrical activity represses AChR gene transcription [reviewed in Changeux et al. (1990)]. Ca^{2+} ions (Fontaine et al., 1987; Klarsfeld et al., 1989) and protein kinase C (Fontaine et al., 1987; Klarsfeld et al., 1989; Huang et al., 1992) have been proposed as second messenger system(s) involved in the coupling between the electrically active plasma membrane and the transcriptional machinery.

Cultured chick embryonic myotubes undergo spontaneous electrical activity resulting in myotube contraction (Fischbach & Cohen, 1973). Blocking this electrical activity with the Na^+ channel blocker tetrodotoxin (TTX) increases the number of acetylcholine receptor (AChR) molecules on the cell surface (Betz & Changeux, 1979; Klarsfeld & Changeux, 1985) and of AChR α-subunit mRNA (Fontaine et al., 1987). Protein kinase C (PKC) has been proposed to be involved in this process since (a) inhibition of its activity with staurosporine leads to an increase in AChR α-subunit expression in myotubes (Klarsfeld et al., 1989) and in denervated muscles (Huang et al., 1992); (b) no additive increase is observed for cultures treated with both TTX and staurosporine (Klarsfeld et al., 1989); and (c) phorbol esters, which activate PKC, inhibit the increase of AChR α-subunit mRNA caused by TTX (Fontaine et al., 1987) and block the up-regulation of AChR expression by staurosporine (Klarsfeld et al., 1989) as well as the expression of a reporter gene under the control of the chicken AChR α-subunit promoter (Laufer et al., 1991). Intramuscular injection of phorbol esters in denervated (electrically inactive) muscle also blocks AChR subunit gene expression (Huang et al., 1992).

Control of tissue-specific expression of several muscle genes relies upon a set of myogenic factors belonging to the basic helix–loop–helix (bHLH) family of transcription factors [reviewed in Olson (1990) and Weintraub et al. (1991)]. These factors, which include MyoD1 (Davis et al., 1987), myogenin (Wright et al., 1989), MRF4 (Rhodes et al., 1989), and myf5 (Braun et al., 1989), activate transcription of several muscle-specific genes through binding to a conserved CANNTG DNA sequence (E box) in their promoter. The AChR α-subunit gene promoter contains two such sites, and cotransfection experiments show that myogenic factors activate transcription of a reporter gene under control of this promoter; besides, these sites are essential for full activity of the promoter (Piette et al., 1990). During the onset of development in the mouse, variations of AChR α-subunit mRNA levels closely follow the levels of the myogenin transcripts (Duclert et al., 1991; Eftimie et al., 1991). Upon denervation, levels of AChR α-subunit mRNA increase in rat (Merlie et al., 1984), chick (Klarsfeld & Changeux, 1985), and mouse (Duclert et al., 1991; Eftimie et al., 1991). Denervation also causes a transient increase in mRNA for all myogenic factors, and the highest increase in myogenic factor mRNA levels is observed with myogenin in both chick (Piette et al., 1992) and mouse (Duclert et al., 1991; Eftimie et al., 1991). Myogenin gene activity declines rapidly upon electrical stimulation of denervated chick skeletal muscle (Huang et al., 1993). Gene knockout experiments also emphasized the essential role of myogenin in the muscle lineage (Hasty et al., 1993; Nabeshima et al., 1993).

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[1] Abbreviations: SPCLN α-BTX, α-bungarotoxin; AChR, acetylcholine receptor; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase; GST–myog, bacterial fusion glutathione *S*-transferase–myogenin protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PKA, protein kinase A; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin.

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Mouse myogenin can be phosphorylated *in vitro*, by either PKC or PKA, and phosphorylation greatly reduces the DNA binding activity of myogenin (Li et al., 1992a,b). These results point strongly to an important role of phosphorylation in the regulation of the transcription-activating properties of myogenic factors.

We have studied the role of myogenic factor phosphorylation in the regulation of AChR gene expression by electrical activity. We used chick embryonic myotubes as a model system and focused our analysis on the effect of electrical activity on myogenin. We have prepared antibodies specifically directed against the carboxy-terminal part of chick myogenin, localized the protein in chick muscle and cultured myotubes, and followed its distribution and posttranslational modifications upon treatment with reagents capable of interfering with muscle activity. By using a highly specific protein kinase C inhibitor, *in vivo* labeling, and immunoprecipitation, we show that myogenin is phosphorylated in electrically active chick myotubes but dephosphorylated in electrically silent myotubes after incubation with TTX. Treatment with a highly specific inhibitor of PKC produces the same effect. Both reagents increase the expression of AChR on the cell surface, and their effects are not additive. Using an assay specific for PKC, we further demonstrate that treatment of cultured myotubes with TTX causes a decrease in nuclear PKC activity. On the other hand, using a DNA binding assay, we show that phosphorylation of a myogenin fusion protein diminishes its binding to an E box present on the AChR α-subunit enhancer. Thus, myogenin phosphorylation via the PKC pathway seems to be a plausible mechanism by which AChR gene expression is repressed in electrically active muscle.

MATERIALS AND METHODS

Antisera and Immunoassays. Hyperimmune rabbit anti-myogenin antiserum was made against the carboxy-terminal peptide 212–227 of chick myogenin (Fujisawa-Sehara et al., 1990). The peptide sequence shares no homology with other myogenic factors. Immunization of rabbits was performed with the peptide coupled with bovine serum albumin as carrier, emulsified with complete Freund's adjuvant and injected subcutaneously at several sites on the back of the animal. Boosting with incomplete Freund's adjuvant was done 3–4 weeks later, and the immune serum was tested for the presence of anti-myogenin antibodies. Hyperimmune rabbit anti-CMD1 (the chick homologue of MyoD) was prepared in the same way against the carboxy-terminal peptide 279–298 of CMD1 (Lin et al., 1989). Immune sera were tested in an enzyme-linked immunoassay with myogenin or CMD1 peptides coated on plastic 96-well plates: the criteria to be met were significant reactivity with the relevant peptide and no reactivity with irrelevant peptides. The selected immune sera were further submitted to a second selection through a microimmunoblot test according to Nghiêm (1988) with both glutathione *S*-transferase–myogenin (GST–myog) and GST–CMD1.

Tissue Culture. Primary cultures of chick myoblasts were prepared as described (Fontaine et al., 1987). Pharmacological agents were added after myotube fusion (4–5 days in culture); cells were contracting intensely at this stage. The protein kinase C inhibitor GF109203X was a kind gift from Dr. J. Kirilovsky (Laboratoires Glaxo, Les Ulis, France); it was used at a final concentration of 0.5 μM. At this concentration, PKC inhibition was maximal, while PKA activity was completely unaffected. Tetrodotoxin was used at 1 μM concentration.

In Vivo Labeling. Primary cultures were labeled with [^{32}P]-orthophosphate (Amersham) at a radioactivity concentration of 0.5 mCi/mL during 6 h in phosphate-free Eagle medium (Sigma). Dishes were rinsed three times with phosphate-buffered saline, and total cell extracts were prepared essentially as in Li et al. (1992b); nuclear extracts were prepared as in Schreiber et al. (1989).

Immunoprecipitation. One milliliter of ^{32}P-labeled extract was precleared with 100 μL of protein A CL-4B beads (Pharmacia) for 1 h and then submitted to immunoprecipitation with anti-myogenin antibodies in the presence of 50 μL of fetal calf serum for 1 h. One hundred microliters of protein A beads was added, and the mixture was incubated for another 1 h. Beads were collected by centrifugation and washed three times with lysis buffer (0.14 M NaCl; Li et al., 1992b) and once with 5 mM EDTA–50 mM Tris buffer, pH 8.5. Immunoprecipitated proteins were boiled in DTT–SDS sample buffer and analyzed by SDS–PAGE. With unlabeled cultures, the immunoprecipitated proteins separated in the SDS gels were transferred to a nitrocellulose sheet and tested with anti-myogenin antibodies. With ^{32}P-labeled cultures, the SDS gels were fixed with 7.5% acetic acid in 5% methanol and then autoradiographed with Kodak X-Omat film. Quantitative data were obtained by scanning the autoradiograms with a Masterscan (Scanalytics, Computer Signal Processing Inc.).

Immunofluorescence. For *in situ* localization of myogenin, 11-day embryonic chick leg muscles were dissected, washed in phosphate-buffered saline (PBS), fixed overnight with cold 4% (wt/v) paraformaldehyde in PBS, sectioned with a microtome (40-μm thickness), and permeabilized with 0.1% Triton X-100 in PBS. Double staining for myogenin and for nuclei was performed: myogenin was detected with anti-myogenin antibodies (dilution 1:500) and fluorescein-labeled secondary antibodies, while nuclei were stained with propidium iodide. The specificity of the immunolabeling was demonstrated by a lack of staining when anti-myogenin antibodies had been preabsorbed with the relevant myogenin peptide. Chick muscle primary cultures were treated with or without TTX (8–24 h). The culture medium was withdrawn, and cells were fixed for 10–20 min with cold 4% (wt/v) paraformaldehyde in PBS. Cells were further processed in the same way as muscle sections for double immunofluorescence staining for myogenin and for nuclei.

Enzyme Activity Measurements. Protein kinase C activity was determined in whole extracts from primary chick muscle cultures using a commercial protein kinase C assay system (Gibco BRL). Extracts were prepared according to the instructions provided by the manufacturer. Protein kinase A activity was determined on the same extracts essentially as described in Toullec et al. (1991), using histone IIa as substrate. In the PKA assay, PKC activity was inhibited by use of the specific peptide inhibitor provided with the PKC kit. PKC activity was also determined in nuclear extracts prepared from control and TTX-treated chick muscle cultures.

Preparation of Recombinant Myogenic Factors. Chicken GST–myogenin and GST–CMD1 proteins were created by fusion of their cDNAs (kind gifts from Dr. A. Fujisawa-Sehara and Dr. B. Paterson) in frame with the carboxy terminus of glutathione *S*-transferase (GST). A commercial expression vector (Pharmacia) was used. The fusion proteins were purified on a glutathione–agarose affinity resin (Sigma). For *in vitro* translation, the chicken myogenin cDNA (a gift of Dr. A. Fujisawa-Sehara) was cloned downstream of the T7 promoter. The protein was produced using a coupled tran

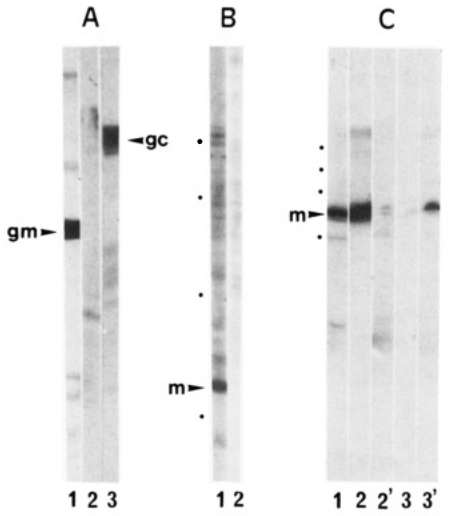

FIGURE 1: Specificity of the anti-myogenin antibodies. (A) In an immunoblot test, the anti-myogenin antibodies stained specifically the bacterial fusion GST–myogenin (lane 1, gm); no immunostaining was observed with a GST–CMD1 protein (the chick homologue of MyoD), another member of the myogenic factor family (lane 2). This GST–CMD1 protein was recognized by anti-CMD1 antibodies (lane 3, gc). (B) The anti-myogenin antibodies stained a polypeptide band of apparent 32–36 kDa mass when reacted with a nuclear extract from chick 11-day embryo skeletal muscle (lane 1, m); the immunoreactivity was no longer observed after preabsorption of the anti-myogenin antibodies with the relevant peptide (lane 2). (C) Autoradiogram of a [^{35}S]myogenin protein obtained by *in vitro* coupled transcription–translation, immunoprecipitated by anti-myogenin or anti-CMD1 antibodies, and then resolved in an SDS gel. The translated myogenin showed the same 32–36 kDa apparent molecular mass (lane 1, m) as myogenin present in muscle nuclear extract. The translated myogenin was immunoprecipitated with anti-myogenin antibodies (lane 2) and not with anti-CMD1 antibodies (lane 3). No myogenin band was detected in supernatant of myogenin immunoprecipitated with anti-myogenin (lane 2′); on the contrary, all the myogenin remained in the supernatant after a parallel immunoprecipitation of myogenin with anti-CMD1 (lane 3′). Molecular mass markers (93, 69, 46, and 30 kDa) are indicated with dots at the left in panels B and C.

scription–translation system (Promega), following the instructions provided.

In Vitro Phosphorylation and Gel Mobility Shift Assay. The GST–myogenin fusion protein was phosphorylated *in vitro* using purified PKC (Promega). Conditions of phosphorylation were those suggested by the protein kinase C kit manufacturers. As a negative control, we used heat-inactivated PKC or performed the reactions in the presence of the peptide inhibitor. The phosphorylated fusion protein was tested without further purification in a gel mobility shift assay, using a ^{32}P-labeled oligonucleotide probe containing one of the two E boxes from the AChR α-subunit promoter (Piette et al., 1990).

RESULTS

Specificity of the Anti-Myogenin Antibodies. A myogenin carboxy-terminal polypeptide characterized by a sequence unique to myogenin among the four myogenic proteins described was chosen as immunogen. Immune sera directed against this polypeptide were selected by ELISA test (see Materials and Methods) and then tested on a GST–myogenin fusion protein by immunoblot. To ascertain the specificity of the antibodies, the selected immune sera were further tested with a nonrelevant myogenic factor. MyoD was taken as representative of the other myogenic factors, and GST–CMD1 (the chick MyoD1 homologue) was reacted with the anti-myogenin antibodies in a sister assay. Figure 1A shows that the antibodies specifically recognized myogenin (lane 1): no labeling was observed with CMD1 (Figure 1A, lane 2), which was stained by anti-CMD1 antibodies (Figure 1A, lane 3).

The anti-myogenin antibodies were next used to probe a nuclear extract prepared from chick embryonic muscle (embryonic day 11). A band of apparent molecular mass 32–36 kDa was stained on a Western blot (Figure 1B, lane 1). Since the sequence-deduced molecular mass of myogenin (Fujisawa-Sehara et al., 1990) was around 23 kDa, we also analyzed the immunoprecipitation of a ^{35}S-labeled myogenin obtained by coupled *in vitro* transcription–translation (Figure 1C, lane 1) with the same anti-myogenin antibodies. The immunoprecipitated ^{35}S-labeled myogenin also showed an apparent molecular mass around 35 kDa (Figure 1C, lane 2), the same as that obtained with the chick embryonic muscle nuclear extract (Figure 1B).

Nuclear Localization of Myogenin in Chick Embryo Skeletal Muscle and in Cultured Chick Embryonic Myotubes. The anti-myogenin antibodies decorated nuclei of embryonic chick muscle fibers (Figure 2A,B) in a specific manner: no labeling was observed either with preimmune serum or with the immune serum previously cleared of anti-myogenin antibodies (Figure 2C). Neighboring cartilaginous tissue was also not labeled. Thus, in the chick, myogenin is localized in the nuclei of embryonic muscle fibers.

Labeling of myotubes cultured in conditions allowing their spontaneous contractions (control myotubes) also resulted in the staining of their nuclei (Figure 2D,E). No detectable change in the subcellular distribution of myogenin was driven by tissue culture. However, myotubes were stained with unequal intensity, and some heterogeneity concerning the expression or the accessibility of myogenin was observed in these cultured myotubes (see Figure 2D,E showing that nuclei of some myotubes were barely or not stained by anti-myogenin antibodies).

Blocking Myotube Spontaneous Electrical Activity by TTX Causes a Decrease in Nuclear PKC Activity. Plausible involvement of PKC in the cellular events linking electrical activity and regulation of AChR α-subunit gene expression has been reported *in vitro* (Klarsfeld et al., 1989; Fontaine et al., 1985). Similarly, PKC has been postulated to participate to the *in vivo* signaling pathway coupling membrane excitation and AChR gene regulation (Huang et al., 1992). To further analyze the role of protein kinases in the coupling between electrical activity and AChR α-subunit gene expression, we prepared extracts from primary cultures of control and TTX-treated chick myotubes and determined their total PKC and PKA activity. As shown in Figure 3A, TTX did produce a decrease of around 16% in total PKC activity. This activity decreased about 60% when nuclear extracts were used (Figure 3B: the PKC activity in nuclear extracts dropped from 100 ± 16% in control to 43 ± 12% in TTX-treated cultured cells). The small increase in PKA activity observed in TTX-treated cultures has already been observed in rat primary cultures and might be due to the increase in cAMP concentration caused by TTX (Chahine et al., 1993). Along this line, Betz and Changeux (1979) have reported that cAMP increases the synthesis of AChR molecules in cultured chick embryonic myotubes.

The PKC Inhibitor GF109203X Enhances AChR Expression. When primary cultures of chick myotubes were treated with the PKC inhibitor GF109203X, a bis(indolyl) maleimide derivative described as a highly specific PKC inhibitor (Toullec et al., 1991), a drastic decrease (over 90%) in PKC activity was observed (Figure 3A). The effect was characteristic for PKC since almost no effect was observed for the PKA activity

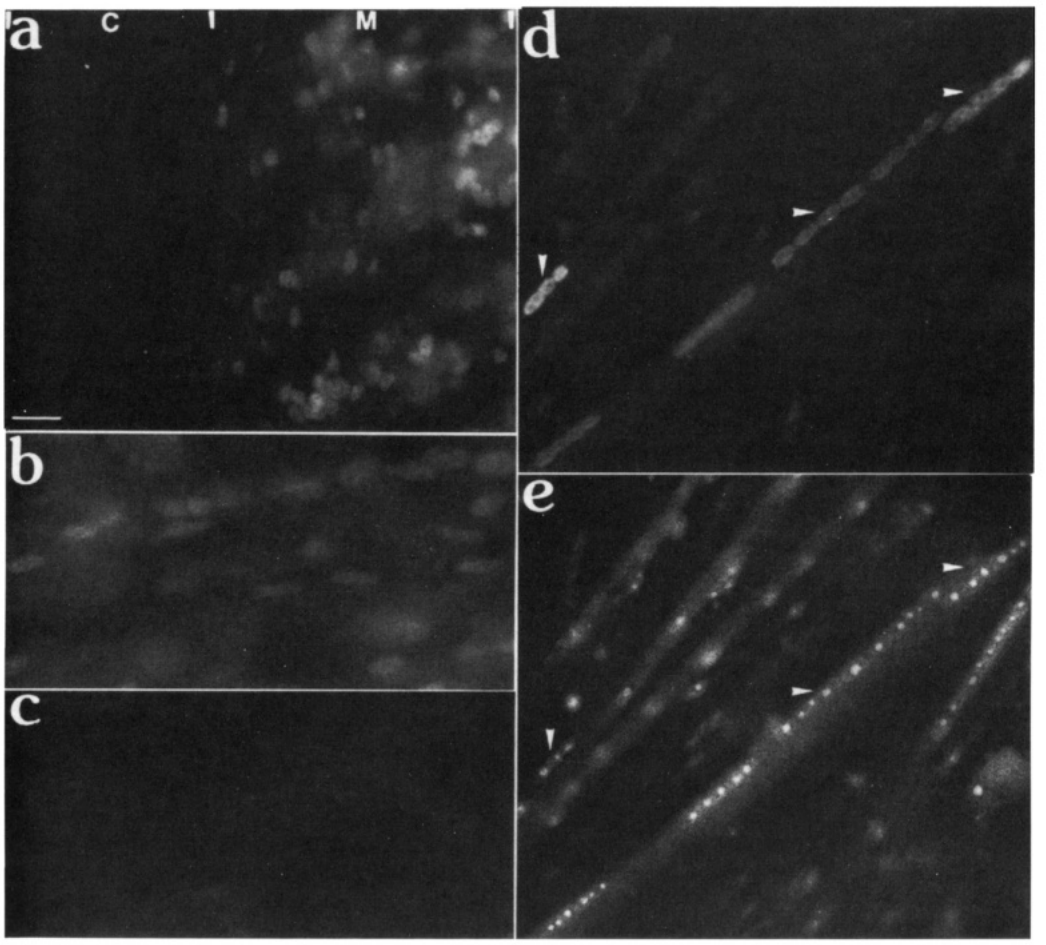

FIGURE 2: Nuclear localization of myogenin in chick embryonic skeletal muscle and chick electrically active (control) cultured myotubes. The anti-myogenin antibodies immunodecorated nuclei of muscle fibers (leg muscle from 11-day chick embryo). (A) The immunolabeling was restricted to nuclei and was muscle (M) specific; neighboring cartilaginous (C) tissue was not stained (bar: 20 μm). (B) Detail of the anti-myogenin immunolabeling of nuclei at higher magnification. (C) The immunostaining was completely eliminated after anti-myogenin antibodies had been removed with the relevant peptide. (D, E) The anti-myogenin antibodies similarly labeled nuclei of cultured chick myotubes from 11-day chick embryo: colocalization of staining with anti-myogenin antibodies (D) and with propidium iodide, a reagent for nuclei (E), was observed. Three examples of colocalization are indicated with arrowheads. Note that nuclei of some myotubes were barely stained or were not stained with the anti-myogenin antibodies; this supports the notion that nuclei from different myotubes can express different levels of myogenin.

present in the same extract (Figure 3A). On the other hand, GF109203X-treated myotubes showed a significant increase in the number of α-bungarotoxin binding sites, hence in the surface AChR molecules. This increase was comparable to that obtained following inhibition of myotube electrical activity with tetrodotoxin (TTX) (Figure 3B). Besides, no further increase in the AChR expression was observed when cultures were treated with both TTX and GF109203X (Figure 3B). Our results are consistent with previous data obtained using staurosporine (Klarsfeld et al., 1989), a PKC inhibitor less specific than GF109203X. They strongly support the conclusion that electrical activity and thus TTX act through the PKC pathway.

Treatment with TTX or Inhibition of PKC Causes Myogenin Dephosphorylation. TTX-treated myotubes showed the same nuclear distribution of myogenin as control myotubes: no TTX-induced translocation of myogenin could be detected by immunofluorescence (data not shown). Myogenin was immunoprecipitated from control and TTX-treated cultures, run on an SDS gel, transferred to a nitrocellulose paper, and reprobed with anti-myogenin antibodies. Myogenin immunoprecipitated from both cultures showed the same apparent molecular mass around 35 kDa (Figure 4, lanes 1 and 2). Moreover, the intensity of their staining by the anti-myogenin antibodies was not drastically different.

Since inhibition of PKC activity by GF109203X caused an increase in the expression of AChR molecules similar to that observed with TTX, we examined the effect of TTX on the phosphorylation state of myogenin. Primary cultures of chick myoblasts were labeled with [^{32}P]orthophosphate for 6 h, and total cell extracts were prepared from labeled cultures. Myogenin was then immunoprecipitated using a specific anti-myogenin antibody. As shown in Figure 5A (lane 1) a ^{32}P-labeled protein was specifically immunoprecipitated from electrically active (control) cultures. This band of around 35 kDa was barely detectable in TTX-treated cultures (Figure 5A, lane 2). On the other hand, the total amount of myogenin in TTX-treated cultures seemed at least equivalent to that present in control cultures as shown by Western blotting (Figure 4, lanes 1 and 2). Thus, the disappearance of the ^{32}P-labeled band caused by TTX treatment does not result from a drastic reduction in the amount of myogenin but rather reflects a dephosphorylation of the protein. In another set of experiments, immunoprecipitation was performed with nuclear extracts: a similar myogenin phosphorylation–dephosphorylation switch was observed from control (phosphorylated myogenin, Figure 5B, lane 1) and TTX-treated (dephosphorylated myogenin, Figure 5B, lane 2) cultures. Results averaged from three independent experiments showed a (5.3 $\pm$ 0.8)-fold increase in the ^{32}P incorporation into nuclear

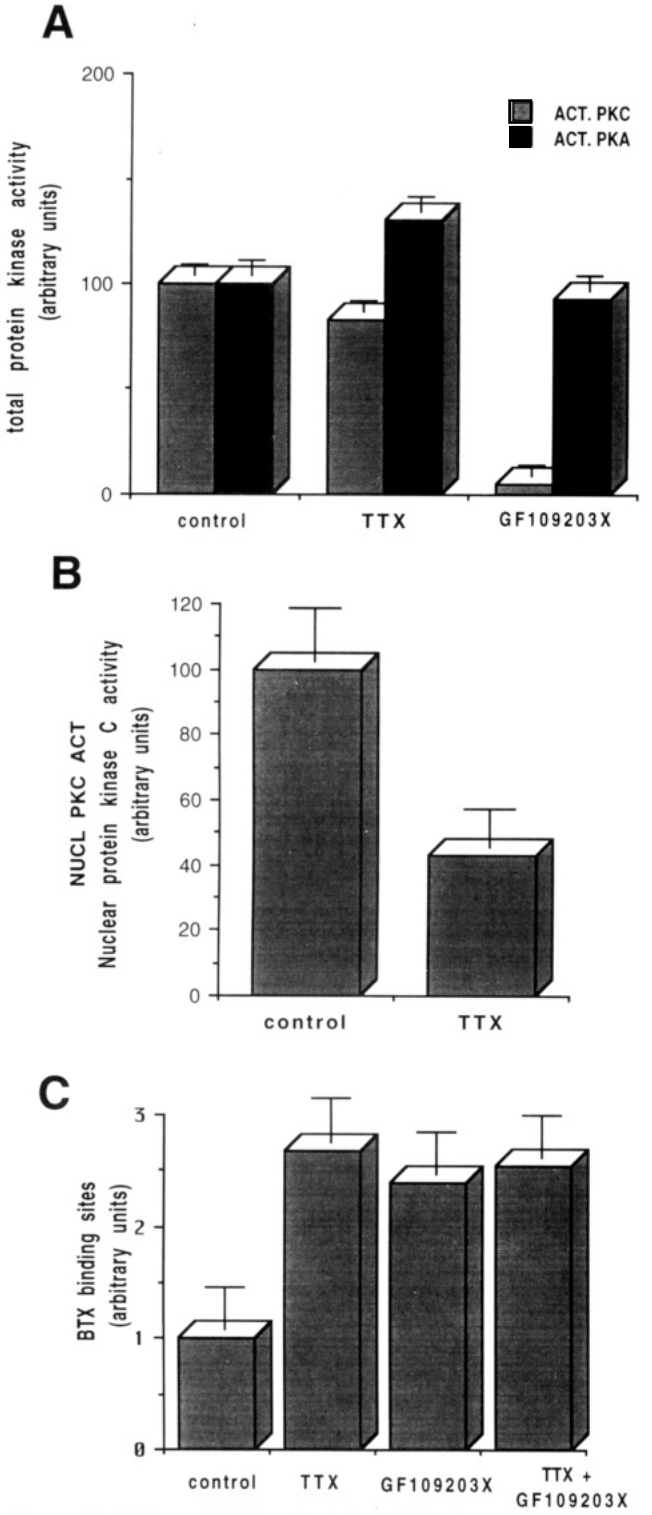

FIGURE 3: Effects of TTX and of the PKC inhibitor GF109203X on AChR expression and protein kinase activity. Results were normalized to the protein content and expressed in arbitrary units. (A) Protein kinase C (PKC) and protein kinase A (PKA) activities were measured in total extracts of control and TTX-treated myotubes (32-h incubation). Two experiments were performed in duplicate. Note the small decrease in total PKC activity (see text for discussion) and the small increase in PKA activity induced by TTX. The PKC inhibitor GF109203X causes a specific decrease in the PKC activity. (B) The TTX-induced decrease in PKC activity was more pronounced when PKC activity was assayed in nuclear extracts. Results are the mean of five experiments, each performed in triplicate. (C) Differentiated chick myotube cultures were treated with the indicated reagents for 32 h of incubation and then tested for surface AChR expression (^{125}I-labeled α-BTX binding sites): TTX and GF109203X induced similar increases in the BTX binding sites. No additivity of the two effects was observed.

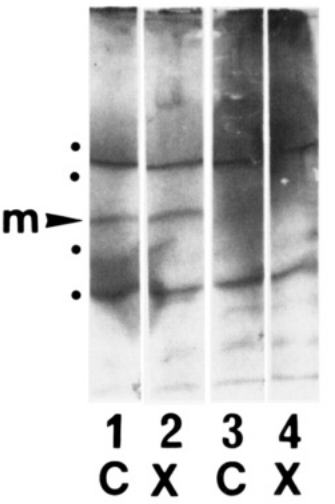

FIGURE 4: Myogenin is present in both control and TTX-treated cultured myotubes. Myogenin was immunoprecipitated from control (C) and TTX-treated (X) cultures with anti-myogenin antibodies prior to (lanes 1 and 2) or after preabsorption of the antibodies with a myogenin peptide (lanes 3 and 4) and then separated on an SDS gel, blotted on a nitrocellulose paper, and probed with anti-myogenin antibodies. Besides the heavy and light immunoglobulin chain bands, the myogenin band of apparent molecular mass around 35 kDa (m) which had been precipitated by anti-myogenin antibodies (lanes 1 and 2) was revealed for both control (lane 1, C) and TTX-treated (lane 2, X) cultures. Note its absence in companion assays when the immunoprecipitating antiserum had been preincubated with the relevant peptide [control (lane 3, C) and TTX-treated (lane 4, X) myotube cultures]. Molecular mass markers (69, 46, 30, and 22 kDa) are indicated with dots at the left.

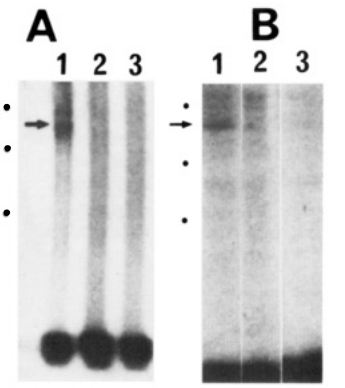

FIGURE 5: Treatment with TTX or PKC inhibitor GF109203X results in dephosphorylation of myogenin in chick myotubes. (A) Total denatured extracts of ^{32}P-labeled control and treated myotubes (6-h incubation) were subjected to immunoprecipitation with rabbit anti-myogenin antibodies and protein A beads and then separated in an SDS gel and autoradiographed. The autoradiogram shows that myogenin precipitated from control myotubes was phosphorylated (lane 1, arrow). No such phosphorylated band was observed either with TTX-treated cultures (lane 2) or with cultures in which the PKC activity had been specifically inhibited by GF109203X (lane 3). (B) The same phosphorylation–dephosphorylation switch of myogenin was observed in another set of experiments when immunoprecipitation was performed with nuclear extracts of ^{32}P-labeled control (lane 1), TTX-treated (lane 2), and GF109203X-treated (lane 3) cultured myotubes. Molecular mass markers (46, 30, and 22 kDa) are indicated with dots at the left. Quantitative data obtained by scanning autoradiograms of nuclear [^{32}P]myogenin immunoprecipitated from control and TTX-treated myotubes are reported in Table 1.

myogenin of electrically active compared to electrically inactive (TTX treated) cultures (Table 1). This is not imputable to a change in myogenin levels (see above and Figure 4) or in ^{32}P incorporation (control/TTX = 97 ± 19% and 105 ± 14% for total cell and nuclear extracts, respectively; Table 1). There was also no difference in the protein content (control/TTX

Table 1: Increase in the Phosphorylated Myogenin Form in Electrically Active (Control) versus Inactive (TTX treated) Myotubes Is Not Due to a General Increase in ^{32}P Incorporation or in Protein Content[a]

cultured myotubes	^{32}P nucl myog	total ^{32}P	nucl ^{32}P	total protein	nucl protein
control/TTX	5.3 ± 0.8	0.97 ± 0.19	1.05 ± 0.14	0.95 ± 0.07	1.12 ± 0.12
no. of expts[b]	3	3	4	4	4
no. of replic/expt[c]	1	2–3	2–3	3–4	3–4

[a] ^{32}P nucl myog = ^{32}P incorporation into immunoprecipitated nuclear myogenin; nucl = nuclear. [b] Number of experiments. [c] Number of replicates per experiment.

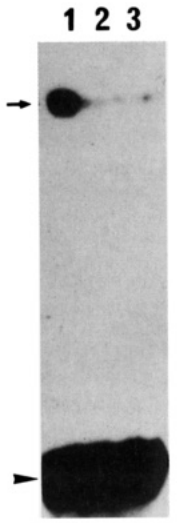

FIGURE 6: Phosphorylation of myogenin by PKC inhibits DNA binding. GST–myogenin was phosphorylated with nonradioactive ATP by PKC and tested for its ability to bind to a ^{32}P-labeled α-AChR oligonucleotide containing a myogenin binding site (gel mobility shift assay). Lane 1: Control GST–myogenin shows a retarded band (arrow) corresponding to binding of the protein to the nucleotide. Lane 2: Specificity of the mobility shift is shown by preincubation of the GST–myogenin with cold oligonucleotide, which nearly abolished the retarded band. Lane 3: The retarded band observed with free GST–myogenin is greatly reduced with the PKC-phosphorylated form (arrowhead: free nucleotide).

= 95 ± 7% and 112 ± 12% for total cell and nuclear extracts, respectively; Table 1). Myogenin dephosphorylation was also observed with sister cultures treated with GF109203X (Figure 5, lanes 3), a pharmalogical agent which specifically inhibits the PKC activity [see above and Toullec et al. (1991)]. The most likely conclusion from these experiments is that nuclear PKC enzyme is involved in the pathway linking electrical activity and the transcription machinery.

Phosphorylated Myogenin Does Not Bind to DNA. Phosphorylation of mouse myogenin has been shown to block its ability to bind to its cognate site in DNA (Li et al., 1992a,b). Inhibition of DNA binding by phosphorylation offers an attractive explanation for our observations in the chicken system. To test this hypothesis, we constructed a bacterial expression vector for chicken myogenin and purified a glutathione *S*-transferase–myogenin fusion protein. The myogenin fusion protein was phosphorylated with purified PKC in the presence of nonradioactive ATP (the control of myogenin phosphorylation was performed with [γ-^{32}P]ATP in a companion tube). Phosphorylated myogenin was tested for its DNA binding activity in a gel mobility shift experiment [a ^{32}P-labeled oligonucleotide containing one of the two myogenin binding sites present in the AChR α-subunit promoter (Piette et al., 1990) was used]. Figure 6 shows a great reduction in the intensity of the retarded band supporting the conclusion that phosphorylation of myogenin greatly diminished its capacity to bind to its specific site in DNA. Taken together, our results strongly support the hypothesis that phosphorylation of myogenin and inhibition of its DNA binding activity might constitute one of the molecular mechanisms by which AChR α-subunit expression is repressed by electrical activity.

DISCUSSION

We have demonstrated that myogenin is a nuclear protein which is phosphorylated in electrically active, and dephosphorylated in electrically inactive, chick myotubes. Several consensus phosphorylation sites (Kemp et al., 1990) are present in chick myogenin. Unfortunately, the low level of ^{32}P-labeled protein precluded determination of the phosphorylation site(s). Phosphorylation–dephosphorylation of transcription factors has been implicated in numerous regulatory processes (Hunter & Karin, 1992). In the case of the AChR, such a mechanism would present the advantage of a rapid response to extracellular (e.g., nervous) signals. The involvement of PKC is supported by the observation that myogenin is dephosphorylated in response to the highly specific PKC inhibitor GF109203X (Toullec et al., 1991). The fact that the increases in the AChR α-subunit expression caused by TTX and GF109203X are similar and not additive strongly suggests that both reagents act through a common mechanism. This is also in agreement with previous data reported by Klarsfeld et al. (1989) on the effect of staurosporine, another inhibitor of PKC, although a less specific one. Huang et al. (1992) also confirmed *in vivo* that PKC is involved in the pathway coupling electrical activity and AChR gene regulation in the chick muscle. With a relative nuclear PKC activity of control over TTX-treated cells around 2.5:1, our results support the hypothesis that TTX treatment causes dephosphorylation of myogenin via a decrease of nuclear PKC activity in the chick muscle cultures. This is in agreement with the increase observed by Huang et al. (1992) in control versus denervated chick muscle. The increase in nuclear PKC activity observed in control over TTX-treated cells (our culture system) as well as between innervated and denervated chick muscle (Huang et al., 1992) is smaller than that observed between electrically stimulated and denervated muscle. This is probably due to the fact that myotubes in their physiological state (innervated muscle) and in culture are both only intermittently electrically active.

Li et al. (1992b) have shown that in transfected 10T1/2 and COS-1 cells PKC phosphorylates and inactivates myogenin through a loss in its DNA binding activity. We have shown in a gel shift assay that, upon phosphorylation by PKC, purified, recombinant chick myogenin loses its ability to bind to its site on the chick AChR α-subunit gene promoter. A similar situation might plausibly occur *in vivo*.

Our results show that myogenin is present in nuclei of both normal and TTX-treated myotubes. At the time of analysis, we did not observe a drastic difference in the staining of myogenin between control and TTX-treated myotubes, either by immunofluorescence or by immunoprecipitation followed by immunoblotting. However, precise quantitation cannot rely on our techniques, and we cannot exclude small differences

in the level of myogenin between control and TTX-treated cultures. Dutton et al. (1993) have reported an increase of both MyoD and myogenin levels in rat primary myotubes, after TTX treatment. Their observations rely only on immunofluorescence staining of normal versus TTX-treated myotubes. It should be noted that while Dutton et al. (1993) observed a uniform nuclear staining for myogenin, we observed a clear heterogeneity in staining intensity: a difference between the anti-myogenin antibody and the propidium iodide staining of myotube nuclei was evident (Figure 2D,E: nuclei of some myotubes were scarcely stained or were not stained with the antibody). In our case, anti-myogenin labeling was performed early (less than 24 h) after TTX treatment of chick myotubes. Total nuclear staining and time of analysis of MyoD and myogenin have not been reported by Dutton et al. (1993). Since the levels of myogenin might vary with time of TTX treatment, our results cannot compare directly to theirs. Laufer et al. (1991) have shown that in chick myotubes cultured in the presence of TPA, an activator of PKC, the level of myogenin mRNA varies greatly with the time of analysis; however, they did not observe any reduction in myogenin mRNA levels prior to that of AChR α-subunit. The differences which exist between the two systems might thus be due to species differences between rodent and bird and/or differences in the time at which analyses have been made. Experimental conditions might also affect the state of spontaneous contractions of cultured myotubes. Edmondson et al. (1991) have shown that myogenin exhibits a short half-life (around 20 min). Duclert et al. (1990) have reported the necessity of *de novo* protein synthesis for the induction of AChR α-subunit gene transcription by TTX. Our results imply that if myogenin is synthetized *de novo* following TTX treatment, the newly synthesized myogenin would not be phosphorylated or would be phosphorylated to a much lower extent.

Taken together, our results strongly support a direct involvement of nuclear protein kinase C in the repression of biosynthesis of the AChR by electrical activity via phosphorylation of myogenin and subsequent inhibition of its binding to DNA and activation of transcription of the AChR subunit genes (at least for the α-subunit). This decrease in DNA binding activity of phosphorylated myogenin leads to a down-regulation of the number of AChR molecules observed in spontaneously contracting cultured myotubes. Huang et al. (1992) have observed an important increase in the activity of PKC following electrical stimulation of chick skeletal muscle. Li et al. (1992b) have shown that fibroblast growth factor inhibits myogenesis through phosphorylation of myogenin and loss in DNA binding to the E box of muscle-specific genes. Since myogenin (Edmondson et al., 1991) and MyoD (Thayer et al., 1989) can autoregulate their expression, myogenin phosphorylation might also lead to a reduction in its gene expression and a decrease in the total amount of myogenin. A model of this type has already been proposed (Kerszberg & Changeux, 1993). Precise quantitation of myogenin will allow further analysis of this possibility. At this stage, we cannot yet conclude whether regulation of AChR gene expression relies exclusively on such a mechanism or also on other myogenic factors present in the nuclei and/or on the contribution of other second messenger pathways such as phosphatases and/or protein kinase A. Chahine et al. (1993) proposed a role for PKA in the regulation of AChR gene expression by electrical activity. The mechanisms by which phosphorylation prevents myogenin binding to its DNA site remain to be explored, as well as the involvement of an eventual translocation of PKC and of the enzyme isoform(s) in the electrically induced phosphorylation of myogenin.

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