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Rapid inhibition of myogenin-driven acetylcholine receptor subunit gene transcription

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In investigating the coupling of depolarization and transcription in skeletal muscle we have focused on how protein kinase C suppresses acetylcholine receptor subunit genes. The activity of acetylcholine receptor subunit promoters in non-muscle cells co-transfected with myogenic factors and E proteins was measured, and their response to protein kinase C activation analyzed. To simplify interpretation of results, gene activities rather than levels of reporter enzymes were assayed, transcriptional effects of phorbol esters were determined, with drug exposures brief enough to preclude kinase depletion, and analysis was carried out with HeLa cells, which are not liable to myogenic conversion. Myogenin, which had been postulated previously to play a role in denervation supersensitivity (Neville *et al.*, *Mol. Cell. Neurobiol.*, 12, 511-527, 1992), was found to be the only myogenic factor whose inactivation kinetics can account for the plasma membrane-protein kinase C-receptor gene cascade observed in intact muscle (Huang *et al.*, *Neuron*, 9, 671-678, 1992).

Introduction

Analysis of upstream flanking sequences of acetylcholine receptor (AChR) subunit genes has revealed the presence of E boxes (MyoD binding sites, MEF-1 or CANNTG motifs) in all promoters investigated to date (Klarsfeld *et al.*, 1987; Baldwin and Burden, 1988; Y.Wang *et al.*, 1988; X.-M.Wang *et al.*, 1990; Gilmour *et al.*, 1991; Numberger *et al.*, 1991; Prody and Merlie, 1991, 1992; Chahine *et al.*, 1992; Jia *et al.*, 1992). These E boxes are components of enhancer-like units which have been shown to be important in the stage- and tissue-specific expression of AChR subunits. In some cases, preliminary evidence indicates that E boxes are also present in regions important for membrane activity-dependent receptor gene regulation (Merlie and Kornhauser, 1989; Merlie *et al.*, 1991; Chahine *et al.*, 1992; Dutton *et al.*, 1993).

What transcription factors act on these targets *in vivo*? E boxes, first identified as protein binding sites in immunoglobulin enhancers (Church *et al.*, 1985; Ephrussi *et al.*, 1985) serve as targets for the helix-loop-helix (HLH) family of transcription factors. Class A (or I) representatives of this family (hereafter designated 'E proteins') include E2-2, also known as ITF-2 (Henthorn *et al.*, 1990; Murre *et al.*, 1991; Sun and Baltimore, 1991), the products of the *E2A* gene, namely E12 and E47 (also known as E2-5 and pan) (Henthorn *et al.*, 1990; Nelson *et al.*, 1990; Murre *et al.*, 1991; Sun and Baltimore, 1991), and HTF4, also known as HEB (Zhang *et al.*, 1991; Hu *et al.*, 1992), whose chicken equivalent, CTF4, we have partly characterized (Tsay *et al.*, 1992). In skeletal muscle, E proteins are believed to heterodimerize with a subset of class B (or II) HLH proteins, the myogenic determination factors MyoD, myogenin, myf5 and herculin (Lassar *et al.*, 1991; for review of myogenic HLH proteins see Olson, 1990; Weintraub *et al.*, 1990; Edmondson and Olson, 1993). These myogenic determination factors (subsequently also referred to as 'M proteins') are crucial in the activation of the genes coding for MCK and other muscle-specific proteins; they may also be responsible for AChR subunit gene activity.

In all AChR promoters investigated, E boxes have been shown to be required for full promoter activity (Piette *et al.*, 1990; Gilmour *et al.*, 1991; Numberger *et al.*, 1991; Wang *et al.*, 1991; Prody and Merlie, 1991; Chahine *et al.*, 1992; Jia *et al.*, 1992). In addition, direct binding and indirect activation studies have revealed that myogenic factors can drive receptor promoter-reporter constructs by acting on the E box elements (Wang *et al.*, 1991; Jia *et al.*, 1992; Prody and Merlie, 1992). We have been interested in the activation and inactivation of receptor genes occasioned by the denervation and subsequent electrical stimulation of chick skeletal muscle. Myogenin message increases substantially upon denervation (Duclert *et al.*, 1991; Eftimie *et al.*, 1991; Neville *et al.*, 1992), and the myogenin gene is rapidly inactivated upon electrostimulation (Huang *et al.*, 1993). These observations, in conjunction with the presumed autocatalytic properties of myogenin (Edmondson *et al.*, 1991), permit the construction of a model in which myogenin plays a crucial role in the control of the expression of the embryonic/extrajunctional or γ type AChR. Theoretical considerations as well as numerous measurements of receptor protein and message levels after treatment with drugs that affect cytosolic calcium levels have implicated calcium as a second messenger or AChR 'shut-off factor' (Changeux and Danchin, 1976; Lømo, 1976; Birnbaum *et al.*, 1980; Pezzementi and Schmidt, 1981; Shieh *et al.*, 1983; Klarsfeld *et al.*, 1989); more recently evidence has been obtained suggesting that activation of protein kinase C (PKC) is part of the signalling pathway (Bursztajn *et al.*, 1988; Klarsfeld *et al.*, 1989; Laufer *et al.*, 1991; Huang *et al.*, 1992).

It has long been known that electrical stimulation of denervated muscle blocks the expression of extrajunctional AChR (Lømo and Westgaard, 1975), but the signals coupling membrane depolarization and gene activity remain poorly understood. We have recently established that PKC is a mediator in this signalling pathway: electrostimulation of denervated muscle, with a delay of 3-4 min, activates nuclear PKC which, in turn, leads almost instantly to the inactivation of the genes that code for receptor subunits

(Huang *et al.*, 1992). It appears as if a factor required for the expression of these genes is inactivated by phosphorylation; that this factor should have autocatalytic properties is suggested by the kinetics of receptor gene expression following denervation and electrical stimulation (Neville *et al.*, 1991). Myogenin, which activates its own promoter, is a plausible candidate, especially since the myogenin gene is much more rapidly silenced by PKC than the genes coding for the other M proteins (Huang *et al.*, 1993). Recently Li *et al.* (1992) have shown that PKCα, the isoform of PKC which is prevalent in skeletal muscle, can phosphorylate (either directly or indirectly), and thereby inactivate, myogenic factors. Their findings fit into a scheme, in which an M protein links kinase and receptor genes, but they do not permit the identification of the specific M protein likely to govern AChR gene transcription, nor a discrimination between direct and indirect actions of the kinase.

We wanted to know whether the inactivation of any of the M proteins by PKC is fast enough to account for the rate of gene inactivation observed *in vivo*. In addition, it was of interest to determine if PKC is likely to act directly or indirectly, e.g. by inhibition of a protein phosphatase. We conclude from our findings, obtained largely by a ribonuclease (RNase) protection version of the transcript elongation assay, that myogenin inactivation by PKC accounts, both qualitatively and kinetically, for the effects of electrostimulation.

Results

Activation of E box-containing promoters by combinations of M and E proteins

To compare the effects of PKC activation on different M proteins we needed a reporter gene construct readily activated by each member of this factor family; in addition, a suitable E protein had to be identified as a common dimerization partner, and reporter gene constructs had to be found that could be driven by a single transfected HLH protein. We therefore transfected four M and two E proteins singly as well as in all possible binary M/E combinations into HeLa cells. Reporter gene constructs contained portions of the 5′ flanking regions from the AChR subunit and myogenin genes (α2kbCAT, α116CAT, γ926CAT, γ275CAT, γ115CAT, δ690CAT, δ207CAT, MG170CAT and MG800CAT), as well as a multimerized E box (E4CAT). Reporter gene activity was measured either indirectly, using CAT assay, or directly, by transcript elongation analysis. Little activity was observed with single factors, with the exception of M proteins acting on γ926CAT and δ690CAT, and of E2-5 activating E4CAT. All M protein/E2-5 combinations were active, to varying degrees, when monitored with any of the reporter constructs; in contrast, M proteins combined with CTF4 were active with all γ promoter constructs and with δ690CAT, but were otherwise fairly inert. As an example of this survey, the activation of the reporter genes with the myogenin/E2-5 driver combination is shown in Figure 1.

Phorbol esters rapidly block myogenin/E2-5-activated genes

The myogenin/E2-5-driven α-subunit promoter, in both short (α116CAT) and long (α2kbCAT) versions, is rapidly inactivated to near control levels upon addition of PMA to the culture medium. δ-subunit and myogenin promoters

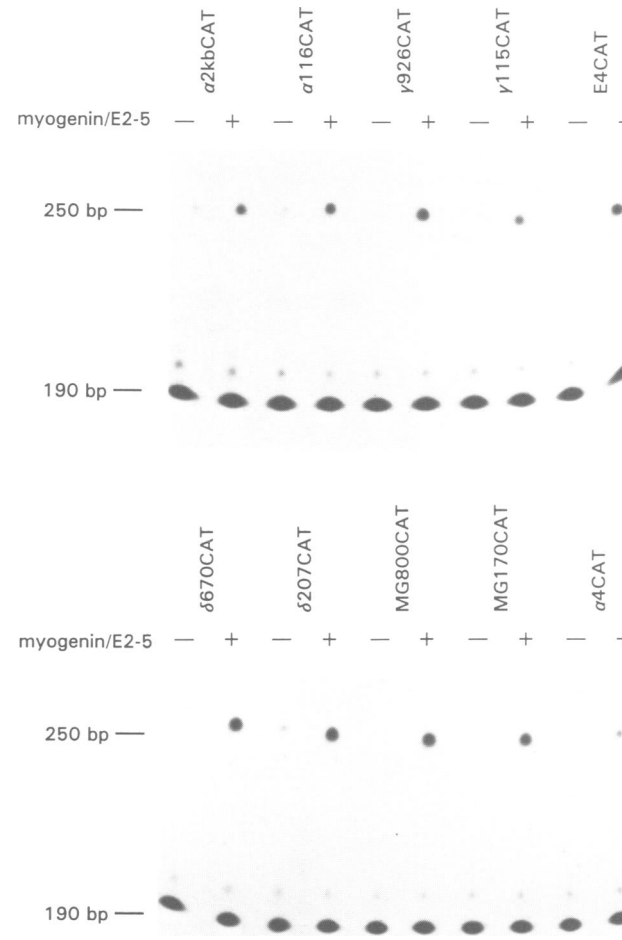

Fig. 1. Activation of E box-containing gene constructs by myogenin/E2-5. The indicated promoter−CAT constructs were transfected with or without myogenin/E2-5 into HeLa cells. 48 h after transfection, nuclei were isolated, and gene activity was determined as described in Materials and methods. The top protected band (250 bp) reflects the strength of individual promoters, while the bottom one (190 bp) reveals the activity of the SV40 promoter (pSV2CAT*Xba*I, internal control).

respond with similar speed, reaching approximately half-inhibition by 5 min, and 80−90% inhibition by 10 min. The inhibition of the γ-subunit promoter proceeds at a somewhat slower pace (Figure 2). All reporter genes return to near control activities within 30 min. The inhibitory effects of PMA on AChR genes resemble the effects of electrostimulation and phorbol ester administration on these genes in live animals (Huang *et al.*, 1992), suggesting that the transfected HeLa cells contain the essential elements required for the signalling pathway that controls the response of AChR genes to phorbol esters in chick skeletal muscle.

M proteins respond differentially to PKC activation

We were also interested in measuring the phorbol ester response of the remaining M proteins. PKC regulation of factors other than myogenin was measured with α116CAT and γ926CAT which are activated by all M protein/E2-5 combinations. In contrast to the complete inactivation seen with myogenin, there was only ~20% and 30% inhibition of the α promoter construct in the case of herculin and myf5 respectively, even after treatment for 30 min; MyoD-driven activity declined by 20% within 10 min, and by an additional

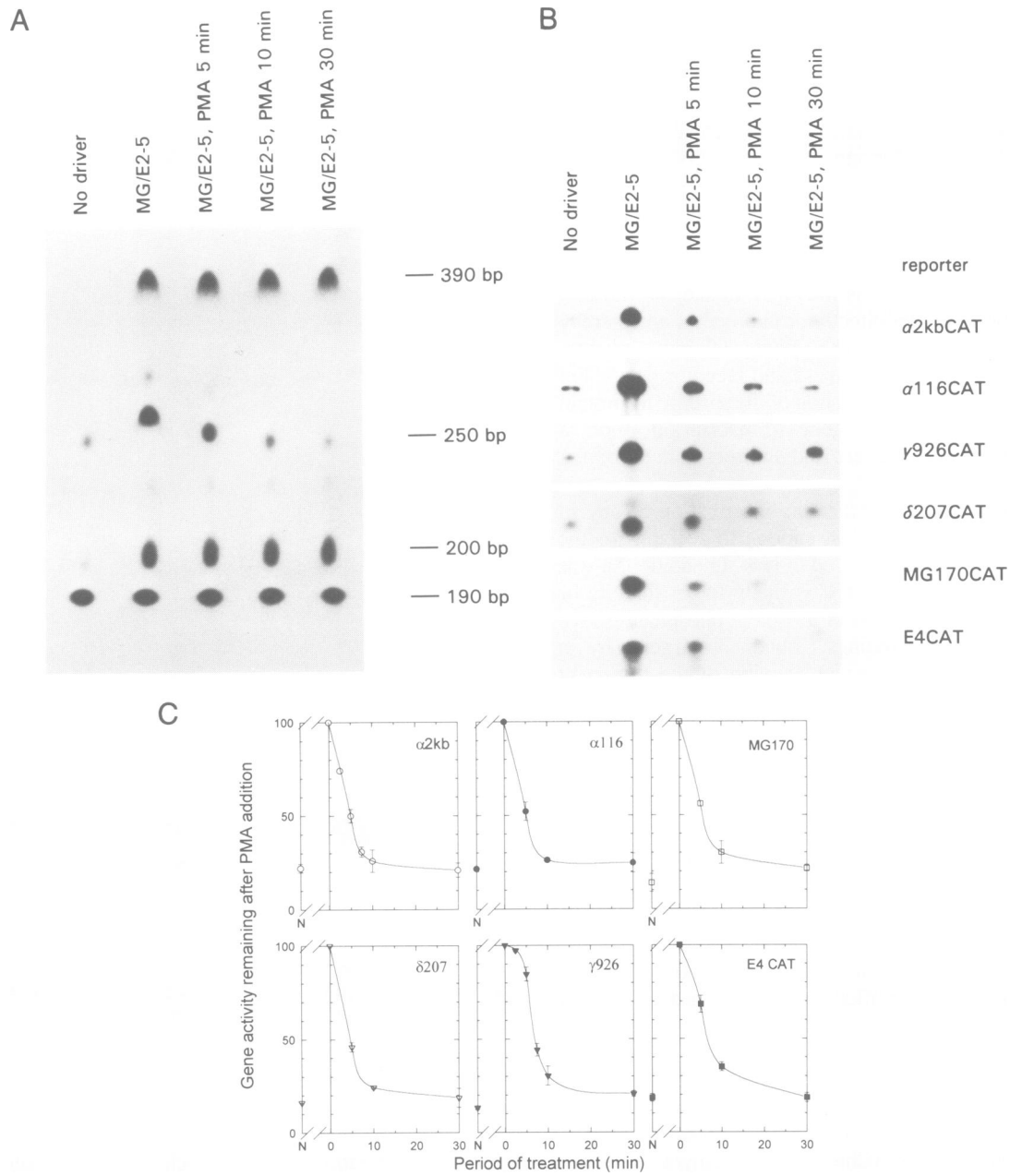

Fig. 2. Response of myogenin/E2-5-driven genes to phorbol esters. Several reporter gene constructs were transfected with or without myogenin/E2-5. Constructs that were activated by myogenin/E2-5 were treated with phorbol ester (PMA) for the indicated periods of time. 48 h after transfection, nuclei were isolated, and gene activities determined. (**A**) Autoradiograph of gene activity analysis for α2kbCAT. The four protected regions, from top to bottom, are fragments of transcripts coding for myogenin (390 bp), α2kbCAT (250 bp), myogenin (200 bp) and pSV2CAT*Xba*I (190 bp). While α2kbCAT is rapidly blocked by phorbol esters, the myogenin transcription rate remains constant. (**B**) Response of α2kbCAT and other promoter−CAT constructs to PMA treatment. Experiments were performed as described in (A); the signals from the top protected region (250 bp) are shown. (**C**) Quantitative analysis of response to PMA treatment. Each data point represents the mean of two to four independent measurements. After correcting for expression of pSV2CAT*Xba*I, results were normalized to the transcriptional activities in myogenin/E2-5-driven promoters. N, reporter only, without a co-transfected driver.

20% upon further drug exposure; similar results were seen with γ926CAT (Figure 3). Thus only myogenin responds as would be expected of the factor involved in the depolarization/gene inhibition cascade *in vivo* (Huang *et al.*, 1992).

Effect of phorbol esters on E protein activity

It is conceivable that PKC blocks myogenin function by phosphorylating its E protein dimerization partner. To test this possibility, the activities of myogenin and of E2-5 were examined individually. In HeLa cells, myogenin can activate γ926CAT without a co-transfected E protein expression vector and is rapidly blocked by PKC activation (data not shown). This is most simply explained as the result of direct myogenin inactivation, but leaves open the possibility that an unknown endogenous E protein, that may serve as a dimerization partner, is inhibited by PKC activation. We therefore investigated the susceptibility of E2-5 in the absence of myogenin. E2-5 is a fairly potent activator of the E4CAT reporter construct in the absence of myogenic factors; upon exposure to PMA the activity of the reporter gene is not reduced, but rather increases, nearly doubling over a period

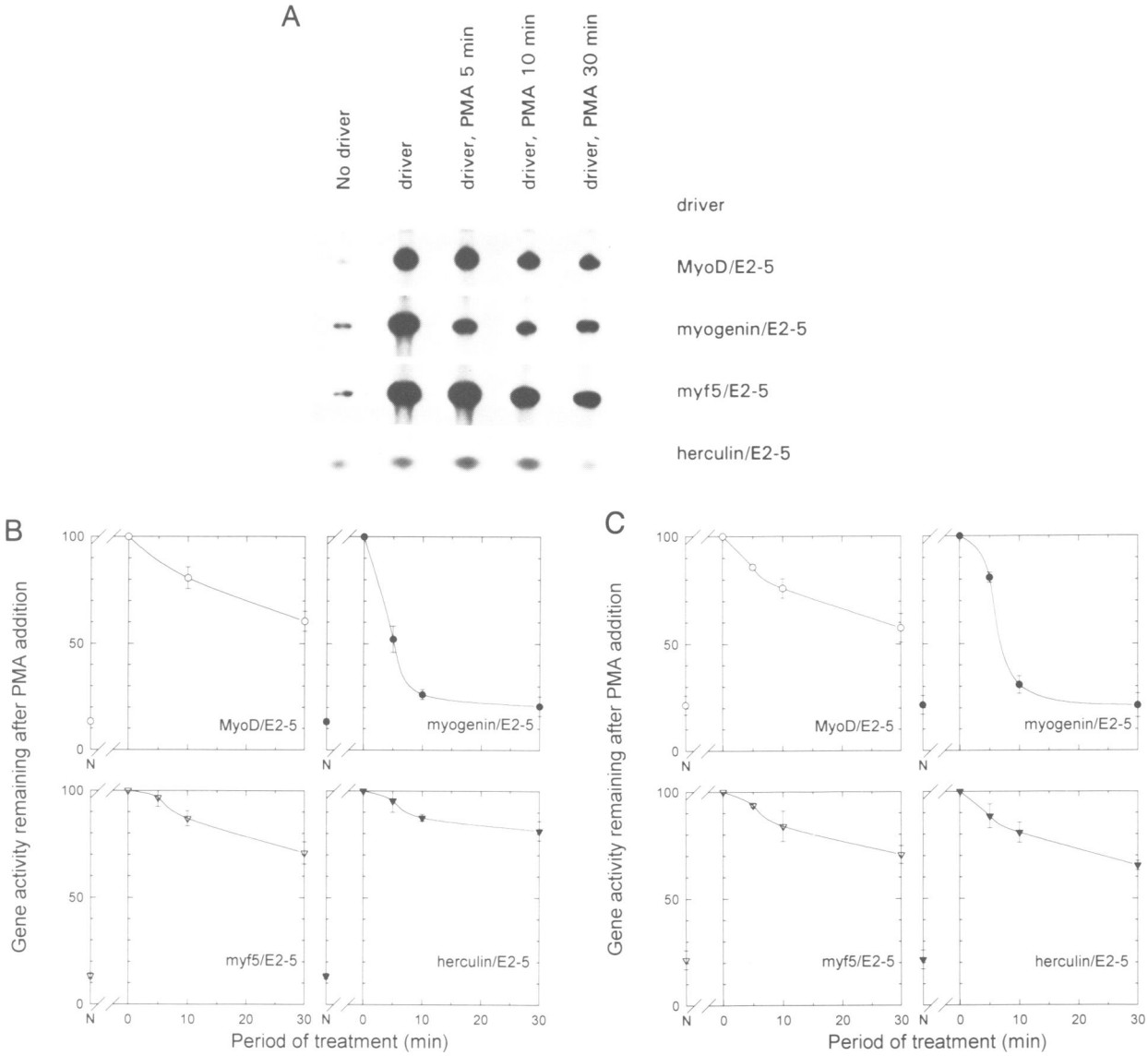

Fig. 3. Kinetics of phorbol ester response of the four M proteins. α116CAT and γ926CAT were transfected with or without the indicated drivers. 48 h after transfection, cultures expressing the reporter gene were treated with PMA for the indicated periods of time, and assayed for gene activity. **(A)** Autoradiograms of RNase protection analysis of α116CAT; the signals from the top protected region (see legend to Figure 1) are shown. **(B)** Quantitative analysis of α116CAT. α116CAT was co-transfected with the four M proteins, and cells treated with PMA for the indicated periods of time. Each data point represents the average of two independent measurements. Results are normalized to the transcriptional activities at t_0. N, reporter only, without a co-transfected driver. **(C)** Analysis of γ926CAT; each data point represents the average of three independent measurements.

of 30 min (Figure 4). These observations suggest that myogenin rather than its dimerization partner mediates gene suppression by phorbol esters; they do not rule out the inactivation of an (as yet unknown) E protein or of an accessory protein that specifically interacts with the M/E complex.

Effect of kinase and phosphatase inhibitors

Although phorbol esters bring about a rapid inhibition of myogenin-dependent gene activity, the action of PKC may be indirect. Rather than targeting the transcription factor itself, the kinase might phosphorylate and thereby inactivate a phosphatase, which in turn could result in the increased phosphorylation of myogenin. As a result of rapid phosphorylation−dephosphorylation reactions, a fraction of the factor would be phosphorylated at any given time. Pharmacological blockade of the phosphatase would therefore be expected to lead to an inactivation of myogenin as fast as, or faster than, the inactivation seen with phorbol esters; conversely, a general protein kinase inhibitor should result in rapid activation. We therefore tested the response of the MG/E2-5 α116CAT system to the administration of staurosporine and okadaic acid, nonspecific inhibitors of protein kinases and phosphatases, respectively. There is little effect of these inhibitors, suggesting that, even if phosphorylation and dephosphorylation of the transcription factor were ongoing, these reactions would be far too slow to explain the phorbol ester effect; appropriate controls demonstrate that the inactivity of staurosporine and okadaic acid is not the result of the drug insensitivity of the HeLa cell enzymes (Figure 5).

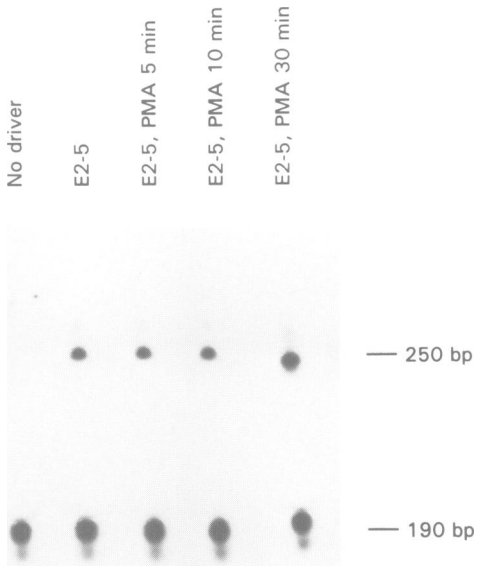

Fig. 4. Effect of phorbol ester treatment on E2-5-driven E4CAT activity. E4CAT was transfected with or without pEMSVE2-5. 48 h after transfection, co-transfected cultures were treated with PMA for the indicated periods of time and assayed for gene activity. The top protected band is due to E4CAT; the bottom band represents activity of the control plasmid (pS2CAT*Xba*I). A second experiment yielded indistinguishable results.

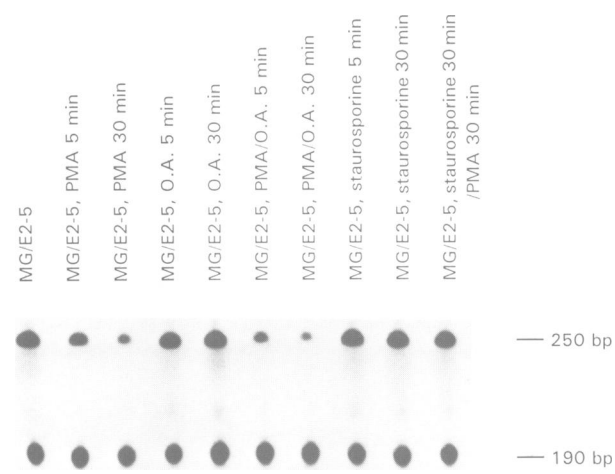

Fig. 5. Effect of kinase and phosphatase inhibitors. α116CAT was transfected with or without myogenin/E2-5. 48 h after transfection, the myogenin/E2-5 cultures were treated with 15 nM staurosporine (STA) or 40 nM okadaic acid (O.A.) for the indicated periods of time; these concentrations suffice to block PKC and PKA (Abdul-Ghani, 1991) and the major forms of protein phosphatase except PP2C (Matsumoto and Yanagihara, 1989). Then nuclei were isolated and the gene activity assayed. The top protected band is due to the activity of α116CAT and the bottom one to that of pSV2CAT*Xba*I. A second experiment yielded indistinguishable results.

Discussion

In the present study we have endeavoured to analyze, in a cultured cell preparation, a link of the proposed signalling cascade responsible for coupling of depolarization and transcription in skeletal muscle. We have chosen HeLa cells for this purpose, primarily because these cells are not converted to the myogenic lineage even by high-level expression of myogenic factors (Weintraub *et al.*, 1989). Consequently, there should be no question whether an observed effect is caused by the transfected factor directly or whether it is a secondary effect of myogenic conversion. [Even if such a question should arise, it would be possible to check for the presence of secondarily induced myogenic factors, since all human MyoD family factors have been cloned (Braun *et al.*, 1989a,b, 1990).] There are added advantages of utilizing HeLa cells: transcription has been intensively studied, and RNA polymerase II and the general transcription factors have been well characterized in this cell type (for a recent review see Zawel and Reinberg, 1993). The presence, activity and drug sensitivity of PKC in HeLa cells is amply documented; it is especially significant that PKCα is the major immunoreactive isoform detected in these cells (Reich and Pfeffer, 1990; Basu *et al.*, 1992). The identity of the PKC isoform involved in depolarization–transcription coupling in skeletal muscle has not been established. However, available evidence suggests that it may be PKCα: (i) the enzyme involved in the control of AChR subunit genes in chick muscle is a conventional, staurosporine-sensitive PKC (Huang *et al.*, 1992); and (ii) the only conventional PKC expressed in skeletal muscle of both mouse or rat is the α enzyme (Osada *et al.*, 1992; Yu *et al.*, 1992).

We have previously investigated gene activity in intact muscle in the minute time range, using transcript elongation assays (Huang *et al.*, 1992, 1993). In order to maintain high temporal resolution in the analysis of transfected cells we have adopted this approach for the present study. This solves two problems: (i) it provides information on gene activity rather than on the amount of a reporter enzyme elaborated as a result of transcriptional and post-transciptional processes; (ii) it completely eliminates the concern about paradoxical effects of phorbol esters. The latter are caused by the preferential degradation of activated PKC: while short-term treatment with PMA and related drugs leads to PKC activation, chronic exposure to the activator results in depletion of the enzyme, i.e. apparent inhibition. For example, in cultured chick muscle cells, phorbol esters cause significant (~80%) reduction in enzyme levels in <8 h (Zhu *et al.*, 1991). Consequently, without actually measuring the amount of PKC, it is difficult, in experiments lasting from more than several hours to less than a day, to attribute the observed result to the activation or disappearance of the enzyme.

Of all factors tested, only myogenin resembles the postulated endogenous transcription factor in the speed and extent of its response to PKC. In fact, the rate of inactivation of the α-subunit, δ-subunit and myogenin genes seen in denervated, electrostimulated chick muscle is very similar to the course of inactivation of reporter genes driven by myogenin in HeLa cells [half-life of ~2–3 min (Huang *et al.*, 1993)]. It appears then that the distal limb of the depolarization–transcription pathway in chick skeletal muscle can be realistically reconstituted in HeLa cells transfected with avian myogenin and avian AChR subunit regulatory regions; it is particularly intriguing that γ926CAT responds less abruptly to PMA than the α-subunit, δ-subunit and myogenin reporter constructs, just as *in vivo* the γ-

subunit gene is inactivated more slowly than other depolarization-susceptible genes (Huang *et al.*, 1992).

A plausible assumption is that myogenin is active in the unphosphorylated form. Phosphorylation/inactivation of the factor could be brought about by PKC acting directly or indirectly, i.e. via activation of another kinase or inactivation of a phosphatase. The latter mechanism might be revealed by demonstrating that a phosphatase inhibitor such as okadaic acid simulates the phorbol ester effect. We observed that okadaic acid minimally affects gene activity, suggesting that myogenic factors undergo little spontaneous phosphorylation and dephosphorylation in the cell. Upon activation of PKC, okadaic acid results in a slighly more noticeable inhibition, as one might expect from a treatment that stabilizes phosphoproteins. Conversely, factor activity remains unaltered in the presence of staurosporine which is the expected result if kinases do not target the factor in untreated cells. We conclude from these experiments that PKC directly targets and thereby inactivates myogenin.

It remains possible that another factor, accessory but necessary for the function of the HLH protein dimer, is the target of PKC activity. Weintraub and his colleagues have recently obtained evidence that rhabdomyosarcoma cells lack such a factor required for the function of MyoD family proteins, which can be supplied by the fibroblast cell line 10T1/2 (Tapscott *et al.*, 1993); this factor may be related to the HIV1 regulatory protein vpr which enables rhabdomyosarcoma cells to differentiate (Levy *et al.*, 1993) or to RB which binds to myogenic factors and also may be required for their proper function (Gu *et al.*, 1993).

Materials and methods

Cell culture and transfections

HeLa cells were grown in 5% calf serum in DME and transfected as described previously (Wang *et al.*, 1990; Jia *et al.*, 1992). Five micrograms each of expression vector and reporter gene construct were co-transfected into cells (cultured in 60 mm dishes), using the calcium phosphate precipitation technique (L.G.Davis *et al.*, 1987). For gene activity analysis, cells were grown for 48 h after transfection, washed with cold 1 mM EDTA in PBS (5 ml/150 mm plate), then scraped and centrifuged at 4°C at 270 *g* for 10 min. The pellet was resuspended in 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1% NP40 and 14 mM mercaptoethanol, placed on ice for 5 min and centrifuged at 220 *g* for 5 min at 4°C. The resulting pellet was resuspended in 1 ml buffer E (50% glycerol, 20 mM Tris pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT and 0.1 mM PMSF) and centrifuged at 13 000 *g* for 40 s at 4°C. The nuclei were resuspended in 20 μl buffer E with 100 U/ml human placenta RNase inhibitor (Boehringer Mannheim, Indianapolis, IN). The nuclei were counted and either used immediately or stored in liquid nitrogen for up to 2 months without detectable loss of activity.

Reporter constructs

The α2kbCAT reporter was constructed by inserting a segment of AChR α-subunit genomic DNA (extending from −1919 to +55 relative to the transcription start site and containing *Bgl*II sites at both ends) into the CAT vector. The *Hin*dIII−*Sam*I fragment of the AChR α-subunit genomic DNA mentioned above (the shortest fully active 5′ deletion mutant comprising 116 bp of upstream flanking sequence) was cloned into the CAT vector to generate α116CAT. δ207CAT was prepared as described earlier (Wang *et al.*, 1990). γ926CAT was the construct reported previously (Jia *et al.*, 1992). A sequence of the chick myogenin upstream region extending from −800 to −1 relative to the translation start site and the 3′-terminal 170 bp fragment thereof, the shortest fully active 5′ deletion mutant, were cloned into the CAT vector to generate MG800CAT and MG170CAT, respectively. E4CAT contains four tandem copies of an oligonucleotide that carries two E boxes (E5+E2) from the IgH enhancer. The pSV2CAT*Xba*I plasmid was obtained from pSV2CAT by deleting the 3′ end of the CAT gene fragment with *Xba*I; the resulting plasmid contains 190 bp of the 5′ end of the gene.

Expression plasmids

In all cases, pEMSVscribe plasmids (R.L.Davis *et al.*, 1987) were used as eukaryotic expression vectors. To generate the chick myogenin expression vector, the full-length cDNA was excised from the Bluescript plasmid PSK$^+$, using *Eco*RI and *Xho*I; after Klenow fill-in it was cloned into the Klenow filled-in *Eco*RI site of pEMSVscribe. To generate chick MyoD, bovine myf5 and rat MRF4 expression vectors, the full-length cDNAs were excised from the CMD-1, pGEM3zf(−)/bmyf5 and pBSMRF4+ plasmids with *Eco*RI and cloned into pEMSVscribe; to generate the CTF4 expression vector, a 4.2 kb cDNA obtained by expression cloning (Tsay *et al.*, 1992) was cloned into pEMSVscribe. The human E2-5 expression vector was prepared by excision from pT7E2-5, using *Hin*dIII/*Bam*HI digestion, followed by Klenow fill-in and cloning into the blunt-ended *Eco*RI site of pEMSVscribe.

Riboprobes

The riboprobe template for CAT was derived from the full-length cDNA cloned into the Bluescript plasmid pSK$^-$, followed by linearization with *Bam*HI; transcription with T7 RNA polymerase yields a probe that protects a region of 250 bp. The probe for chicken myogenin was derived from a denervated muscle cDNA library (protected regions of 200 and 390 nt).

Transcript elongation analysis

Transcript elongation was measured as described previously (Huang *et al.*, 1993). Briefly, 10^7 nuclei were incubated with 150 μCi of [^{32}P]UTP (3000 Ci/mmol) at 26°C for 45 min in 100 μl of reaction buffer [300 mM $(NH_4)_2SO_4$, 100 mM Tris (pH 7.9), 4 mM $MgCl_2$, 4 mM $MnCl_2$, 200 mM NaCl, 0.4 mM EDTA, 0.1 M phenylmethylsulfonyl fluoride, 1.2 μM dithiothreitol, 1 mM NTP (except UTP), 29% glycerol and 10 mM creatine phosphate]. Unlabelled UTP was added to a final concentration of 150 μM. Nascent transcripts were purified through a Sephadex G-50 spun column, followed by TCA and ethanol precipitations. For hybridization, aliquots containing a constant amount of radiolabel (10^6 c.p.m.) were incubated with ~0.1 μg of antisense RNA probe at 52°C; RNase A digestion and electrophoretic analysis followed the procedure of Melton *et al.* (1984). Results were quantified using a Beta scanner (Ambis, San Diego, CA) and visualized by autoradiography; gene activities were normalized for pSV2CAT*Xba*I expression.

Enzyme assays

Cells were assayed for CAT and β-galactosidase activity as described previously (Wang *et al.*, 1990).

Drugs and reagents

Staurosporine and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St Louis, MO); okadaic acid was a product of Calbiochem (La Jolla, CA).

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