Phorbol Esters Selectively Downregulate Contractile Protein Gene Expression in Terminally Differentiated Myotubes Through Transcriptional Repression and Message Destabilization

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Abstract. Chronic exposure of differentiated avian skeletal muscle cells in culture to the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (PMA), results in the selective disassembly of sarcomeric structures and loss of muscle-specific contractile proteins, leaving cytoskeletal structures and their associated proteins intact. We demonstrate here that these morphological and biochemical changes are accompanied by dramatic and selective decreases in the level of the mRNAs that encode the contractile proteins. We measured the effects of PMA on the transcriptional activity and mRNA stability of four contractile protein genes (α-cardiac and α-skeletal actin, cardiac troponin C [cTnC], and myosin light chain 1f [MLC1f]) and two nonmuscle genes (β-cytoplasmic actin and the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). The transcriptional activity of the α-cardiac actin and cTnC genes dramatically decreased by 8 h after the addition of PMA, while other muscle and nonmuscle genes examined showed no change. Pulse-chase experiments of in vivo labeled RNA showed significant reductions in mRNA half-lifes for all the contractile protein mRNAs examined, while the half-lifes of β-actin and GAPDH mRNA were unchanged. All of the above effects occurred under conditions in which cellular protein kinase C (PKC) levels had been reduced by >90%. The fact that many of the contractile protein genes remained transcriptionally active despite the fact that the cells were unable to accumulate their mRNAs to any significant extent indicated that the treated cells were still committed to skeletal muscle differentiation. The selective changes in the stability of the contractile protein mRNAs suggest that the control of mRNA stability may be part of the normal regulatory program of skeletal muscle differentiation and that this control may be linked to the integrity of the contractile apparatus and mediated by second messenger pathways involving PKC activation.

Myogenesis follows a succession of developmental stages, each characterized by distinct morphological events and unique patterns of gene expression. These stages are the establishment of a committed cell lineage (determination), the expression of the muscle phenotype, which is marked, in part, by the accumulation of specific structural proteins (differentiation), and the further specialization of the muscle cell through changes in muscle-specific protein isoform expression (maturation). Muscle development in culture has provided a convenient experimental system in which to study some of the regulatory mechanisms associated with these different stages (reviewed in Schwartz and Stone, 1983; Crow, 1987). In this system, committed mesenchymal cells, called myoblasts, proliferate, then eventually withdraw from the cell cycle and differentiate. Differentiation involves two separable phases: biochemical differentiation, which results in the repression of subsets of non-muscle genes and induction of muscle-specific gene expression (Schwartz and Rothblum, 1981; Hayward et al., 1988) and morphological differentiation, in which the mononucleated myoblasts fuse to form multinucleated myotubes. The result is a rapid and extensive accumulation of different muscle proteins and their assembly into the organized structure of the sarcomere. The specialization of the contractile apparatus that is part of the process of muscle maturation and that involves the replacement of one contractile protein isoform for another occurs to only a limited extent in cultured muscle cells. The cells are arrested at a stage that closely resembles early embryonic development in terms of isoform expression (Hayward and Schwartz, 1986; Van Horn and Crow, 1989) and are, presumably, awaiting signals that are normally provided during late embryonic life, hatching, or birth.

Both the biochemical and morphological differentiation of muscle are sensitive to a variety of chemical and biological agents. A number of these interfere directly or indirectly with the ability of the calcium ion to act as an intracellular messenger. Among these are the tumor promoting phorbol esters, such as 12-*O*-tetradecanoyl phorbol-13-acetate (PMA),

which act in the cell to alter Ca^{2+}-sensitive, phospholipid-dependent protein kinase C (PKC)[1] activity (Witters and Blackshear, 1987; Bazzi and Nelsestuen, 1989). A number of groups have shown that PMA uncouples muscle cell differentiation at various levels. For example, PMA stimulates the replication of myoblasts, while inhibiting the biochemical differentiation and fusion of some myoblast populations (Cohen et al., 1977; Cossu et al., 1982; Croop et al., 1982; Farzaneh et al., 1989). When added to fully differentiated muscle cells, PMA leads to the separation of the I-Z-I complex from the thick filaments and the subsequent formation of 3-μm cortical actin-containing bodies composed of sarcomeric α-actin and α-actinin. This is then followed by the rapid degradation of the protein components of the thin filament and sometime later by the degradation of the proteins of the thick filament (Lin et al., 1987). The specificity of this effect for sarcomeric structures is exemplified by the fact that α-actin-containing thin filaments of the sarcomere are rapidly degraded in PMA-treated muscle cells, while β- and γ-actin-containing thin filaments of the cytoskeleton remain intact. These effects of PMA are fully reversible upon its removal (Lin et al., 1989).

The above effects of PMA are of interest for a number of reasons. The precise cataloging of the morphological events that occur after the addition or removal of PMA has and will continue to contribute significant insights into the processes of sarcomere assembly, while the selectivity of PMA's action on the proteins of the contractile apparatus points to underlying control mechanisms that may serve to regulate the cytoplasmic abundance of the contractile proteins and their mRNAs. The well-established link between phorbol esters and PKC activation further suggests that PKC could be the intracellular messenger controlling this regulatory pathway. We therefore sought to determine the level of gene regulation at which PMA exerted its selective effects on the expression of muscle genes and the accumulation of their products. We examined whether PMA exerted a differential effect on the level of muscle-specific mRNA accumulation and whether that effect was due to changes in the transcriptional activity of the genes encoding the contractile apparatus and/or the stability of their encoded mRNAs. Furthermore, since persistent activation of PKC by PMA has been reported to accelerate the degradation of PKC and its loss from the cell (Ballester and Rosen, 1985; Hepler et al., 1988), we measured the changes in PKC accumulation and activity that accompanied the changes in muscle-specific gene expression. Our results demonstrate that treatment of primary avian muscle cell cultures with PMA results in an extensive down-regulation of cellular PKC levels and activity. Coincident with or occurring after this decrease, the muscle-specific mRNAs that encode sarcomeric proteins are selectively destabilized and depleted from the cells. This accelerated loss of muscle-specific mRNAs was effectively blocked by inhibitors of RNA transcription and/or protein synthesis. The effects of PMA at the transcriptional level were even more selective in that it inhibited the transcriptional initiation of some muscle-specific genes while not affecting that of non-muscle genes or other muscle-specific genes.

1. *Abbreviations used in this paper*: AraC, cytosine arabinoside; MLC, myosin light chain; PCR, polymerase chain reaction; PKC, protein kinase C.

Materials and Methods

Primary Muscle Cell Cultures

Myoblasts were isolated from embryonic day 11 or 12 chicken embryonic pectoral muscles as described previously (Hayward and Schwartz, 1986). Cultures were established by either mechanical or enzymatic (trypsin) means of cell dissociation. Both methods yielded the same results in all the assays described below. Myoblasts were plated onto type I collagen-coated plastic dishes at a cell density of $5 \times 10^4/cm^2$ and grown in Dulbecco's Minimal Essential Media (DMEM, high glucose; Gibco Laboratories, Grand Island, NY) supplemented with 10% preselected horse serum, 2.5% chick embryo extract, and 50 μg/ml gentamycin. Media was then changed every 48 h. 3-day-old cultures were treated for 24 h with 5 μM cytosine arabinoside (AraC) to kill replicating cells. This treatment resulted in cultures with <1% of the nuclei in cells other than myotubes.

Purification and Analysis of Total Cellular RNA

Total RNA was isolated from cell cultures by the guanidine isothiocyanate procedure (Chirgwin et al., 1979) and fractionated on 1% agarose-formaldehyde gels after denaturation in methylmercury hydroxide. The RNA was then electrophoretically transferred to Nylon membranes (Duralon; Stratagene Cloning Systems, San Diego, CA) in 1× Tris-acetate-EDTA buffer at 6 V/cm for 4 h. The transferred RNA was then covalently cross-linked to the Nylon by ultraviolet irradiation (120 mJ). The blots were prehybridized at 45°C for 2–4 h in 50% formamide, 5× Denhardt's solution (Sambrook et al., 1989), 0.5% SDS, 0.1% Na_4PO_7, 50 mM Tris HCl, pH 8, 0.5 mM EDTA, and 200 μg/ml denatured salmon sperm DNA. Hybridization was performed in a buffer of the same composition containing 5×10^5 to 1×10^6 cpm/ml of radiolabeled probe.

cDNA Probes

cDNA probes were used to measure contractile protein mRNAs. For the actins, DNA probes to the 3′ untranslated regions of chicken α-skeletal, α-cardiac, and β-cytoplasmic actin were generated by polymerase chain reaction (PCR) and then radiolabeled by the random priming method (Feinberg and Vogelstein, 1983). The fast alkali myosin light chain (MLC) probe was a randomly primed double-stranded 300-bp EcoRI-StyI fragment of the full-length chicken MLC1f cDNA, pGemFLC1 (Crow, M., unpublished data). This restriction fragment contained sequences from the 5′ end of the cDNA that reacted only with MLC1f and not with the related MLC3f mRNA. The cardiac troponin C probe was a randomly primed double-stranded 800-bp BamHI-HindIII fragment from the cDNA clone, pl3 (Putkey et al., 1987) and the GAPDH probe was a 1,200-bp PstI fragment from the chicken cDNA clone, pGAD28 (Dugaiczyk et al., 1983).

Nuclear Run-on Transcription Assays

Relative transcriptional activity was assessed by nuclear run-on analysis. MLC1f, cTNC, and GAPDH transcription were measured using the cDNA probes described above. Actin gene transcription was measured using either the full-length double-stranded cDNA probes pAC269 (Schwartz et al., 1980) for skeletal α-actin and pAC1 for β-cytoplasmic actin (Cleveland et al., 1980) or a genomic clone, pAC7.5, for cardiac α-actin (Chang et al., 1984). These probes were alkali denatured, neutralized, and 1-μg portions dot-blotted onto nylon membrane (Biotrans; ICN, East Hills, NY) in the presence of 5.4× SSC. De novo nuclear RNA transcripts were assayed according to the procedure of Linial and co-workers (1985). Nuclei from cultured muscle cells were isolated by homogenization of the cultures in 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5% NP-40, and 0.5 M sucrose. The released nuclei were pelleted and homogenized again in the above solution without NP-40 and sucrose. The pellet was suspended in 4 pellet volumes of storage solution (40% glycerol, 50 mM Tris-HCl [pH 8.3], 5 mM $MgCl_2$, and 0.1 M EDTA) and then frozen and stored in liquid nitrogen until needed.

The run-on reaction consisted of thawed or freshly prepared nuclei, run-on buffer (5 mM Tris-HCl [pH 8.0], 2.5 mM $MgCl_2$, 150 mM KCl, and 0.25 mM of ATP, GTP, and CTP) RNAse inhibitor, and 120 μC of [^{32}P]UTP (sp act >800 Ci/mmol; Amersham Corp., Arlington Heights, IL) incubated at 30°C for 15 min. After incubation, DNA was removed by DNAse 1 digestion (5 min at 37°C) and protein removed by proteinase K digestion (30 min at 50°C) followed by phenol/chloroform extraction. Nuclear RNA was then partially cleaved and denatured in 0.2 M NaOH (10 min

at 4°C), neutralized, and precipitated in 2.5 vol of ethanol. This labeled RNA was added to a hybridization solution consisting of 50% formamide, 1.5× SSPE (0.27 M NaCl, 0.015 M Na_2PO4 [pH 7.0]), 1.5 mM EDTA, 1% SDS, 0.5% nonfat dry milk, and 0.2 mg/ml *Escherichia coli* tRNA at $\sim 10^7$ cpm/ml. Since sequence mismatches between the different actin isoform mRNA species would be expected to result in a difference in Tm of only 10–13°C, hybridization was carried out at 50°C for 48 h. These stringent conditions allowed for the use of full-length probes with minimal (<5%) cross-hybridization (Chang et al., 1984). After hybridization, the membranes were washed at increasing stringencies, with a final wash in 2× SSC, 1% SDS at 60°C. The dot blots were then rinsed at room temperature in 0.1× SSC, blotted dry, and exposed to x-ray film for 3–5 d at −70°C with an intensifying screen. Autoradiographs were quantitated by soft-laser densitometry after preexposing the x-ray film to an OD A_{540} of ~0.1 as indicated (Laskey and Mills, 1975) and were calibrated by eluting prebound ^{32}P-labeled probe. Exposure of the film was limited so that no spot developed an absorbance >1.5 OD_{540}. The autoradiograms shown in Fig. 2, 3, and 5 were purposefully overexposed for publication.

Measurement of mRNA Half-Lives

The rate of mRNA degradation in PMA-treated and untreated (control) cultures was measured by an extended pulse-chase method using [^{3}H]uridine. Total RNA in muscle cell cultures was labeled to near equilibrium by a 48-h incubation in media containing 0.2 mCi/ml of [^{3}H]uridine ([5,6 ^{3}H]uridine, sp act 36.5–48 Ci/mmol; New England Nuclear, Boston, MA) starting on day 3 after plating. Under these conditions, significant label could be incorporated into the most stable of the contractile mRNAs, e.g., MLC1f. A 100-mm culture typically incorporated $\sim 2 \times 10^7$ cpm or 7.4×10^7 dpm (efficiency of counting = 27%) using this procedure. After labeling, the cells were washed in DMEM and the [^{3}H]uridine chased by incubating the cells in DMEM containing 10 mM uridine and 10 mM cytidine for 2 h at 37°C. The media was then changed to that normally used to maintain muscle cell cultures (see formulation above) with the addition of 1 mM uridine and 1 mM cytidine. Cells were then maintained in this media for the duration of the experiment. No significant different was observed in the total RNA or the specific activity of the radiolabeled RNA (cpm/μg total RNA) between control and PMA-treated cultures. The extreme stability of total RNA (i.e., mostly rRNA) is consistent with previous reports of rRNA stability in exponentially growing cells (Abelson et al., 1974; Emerson, 1971) and in, at least, one muscle cell line (Krauter et al., 1979).

Approximately 5 h after beginning the chase, PMA (75 ng/ml) was added to experimental cultures, while only the vehicle for PMA (DMSO, 0.05% final concentration) was added to control cultures. RNA was then isolated from the cultures by the guanidine isothiocyanate procedure (Chirgwin et al. [1979]) at various times after PMA was added. cDNA and PCR probes were used to measure the relative level of specific tritiated mRNAs. These probes were bound to nylon membrane (Duralon; Stratagene Cloning Systems) by UV cross-linking (254 nm, 120 mJ), hybridized with tritiated RNA at 1×10^6 cpm/ml, and washed as described for the nuclear run-on assays. The hybridized RNA was then digested with pancreatic RNAse and exposed to x-ray film. Background hybridization was assessed by binding of labeled RNA to pGEM3zf(+) and was on the order of 0.5–2 ppm input RNA. Quantitation was performed either by laser densitometry or by direct counting of tritiated RNA bound to the Nylon membrane following elution with 1N NaOH. For quantitation by densitometry, the filter was treated with spray scintillant (EnHance, New England Nuclear) and the x-ray film preflashed to an optical density at 540 nm of ~0.1. The amount of hybridized RNA at any given time ($t > 0$) during the chase was then expressed as a percentage of hybridized counts at the time 4 h after initiation of the chase, which was designated $t = 0$. Equivalent results were obtained with either method of quantitation.

Measurement of PKC Levels and Activity

The relative level of total PKC protein was determined by phorbol ester binding as described previously (Neidel et al., 1983; Jaken, 1987). The binding characteristics of ^{3}H-phorbol 12, 13-dibutyrate [PdBu] (18–20 Ci/mmol; Amersham Corp., Arlington Heights, IL) were first determined by Scatchard analysis. Cells were plated onto collagen-coated 6-well plates (Costar, Cambridge, MA) and nonmuscle cells were removed 3 d later by AraC treatment (see above). Increasing amounts of labeled PdBu in the range of 5–300 nM were incubated with cells at 37°C for 30 min in DMEM containing 10 mg/ml BSA. Preliminary experiments indicated that both specific and nonspecific binding at this temperature was linear up to 60 min. Nonspecific binding was determined for each concentration of labeled PdBu as the amount of label that remained bound in the presence of 20 μM unlabeled PdBu. Specific binding was then determined by subtracting nonspecific from total binding. The efficiency of scintillation counting under the conditions of these experiments was ~37%.

To determine the level of PKC expression by phorbol binding after chronic PMA exposure, the cells were first extensively washed with media and then incubated for 1 h in DMEM-BSA before labeled PdBu in DMEM-BSA was added. This additional incubation period was necessary to remove all traces of PMA, which could significantly interfere with the binding of radiolabeled phorbol dibutyrate since, at equimolar concentrations, PMA was an effective competitor of PdBu binding. If, after adding PMA for 1–2 h, the cells were extensively washed and incubated as described above, ~85–90% of control ($t = 0$) PDBu binding could be retrieved. The failure to actually retrieve 100% of the binding may reflect the failure to wash out all traces of PMA or a PMA-mediated reduction in PKC levels occurring during the period of the washes and incubations.

Changes in total PKC enzymatic activity were also measured to corroborate the result of phorbol binding. Total activity was measured as the sum of the activities from the cytoplasmic and particulate cell fractions, which were prepared according to a modification of the procedure of Martelly and co-workers (1989). Cells in a 100-mm dish were rinsed with Hanks' solution, removed from the plate by scraping in 0.5 ml of 20 mM Tris HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, and then homogenized in a Dounce homogenizer. The homogenate was then spun at 100,000 *g* for 1 h to separate it into soluble and particulate fractions. The supernatant or soluble fraction was diluted to a protein concentration of 0.5 mg/ml. The pellet was resuspended in the homogenization buffer containing 0.1% Triton X-100 and then spun again. The resulting supernatant was reserved as the particulate fraction. Both soluble and particulate fractions were applied to DEAE-Sephadex columns (0.4 × 1 cm) equilibrated with 20 mM Tris HCl (pH 7.5), 0.5 mM EGTA, 0.3 mM EDTA, and 10 mM β-mercaptoethanol. The columns were washed with 10 vol of the equilibration buffer and PKC eluted with 0–0.3 M NaCl gradient. Fractions (0.175 ml) were collected and 20–50 μl of each assayed for PKC activity. PKC activity was assayed using a commercially available kit (Amersham Corp.), which measures the incorporation of ^{32}P from [^{32}P]-γATP into a synthetic peptide substrate for PKC. Specific PKC activity was expressed as the difference between ^{32}P incorporation in the presence and absence of exogenous calcium, 40 μg/ml phosphatidylserine, and 10 ng/ml PMA.

Results

PMA Selectively Represses Skeletal Muscle Gene Expression in Cultured Myotubes

When the phorbol ester PMA was added to chicken muscle cell cultures 4–5 d after plating, the cells failed to assemble any organized sarcomeric structures. After 24 h of exposure to PMA, the cells lost their elongated appearance and were visible as "myosacs" with the multiple nuclei of the cells aggregated in the center (see Fig. 1 and Lin et al., 1987). The effect of PMA on muscle-specific contractile protein gene expression was then evaluated over time by Northern blotting analyses. Fig. 2 shows the results of a typical analysis in which total RNA isolated from PMA-treated and control cultures was hybridized with radiolabeled probes to fast skeletal muscle MLC1f, skeletal α-actin, cardiac α-actin, cardiac troponin C, β-cytoplasmic actin, and GAPDH mRNAs. Within 6 h after the addition of PMA, there was a large reduction in the steady-state accumulation of those mRNAs encoding the proteins of the thin filament (i.e., skeletal and cardiac α-actins and cardiac troponin C); by 18 h, the level of these mRNAs had decreased to ~5–25% of their control values. The fast MLC1f mRNA, which encodes a component of the thick filament, showed a more gradual decline in content and was reduced to only ~60% of its control value at 18 h. By 24 h, the level of this mRNA was 25% of its control and at 48 h, <5%. In marked contrast, the mRNAs encoding

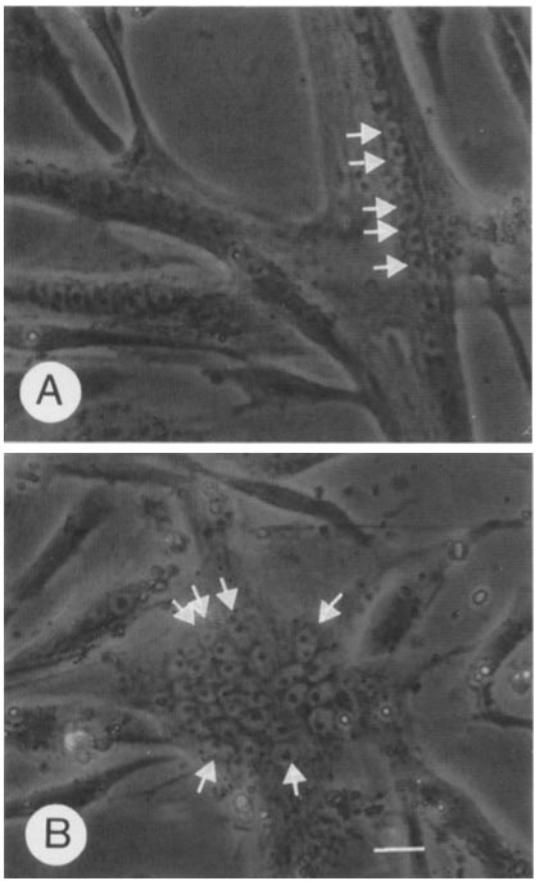

Figure 1. Phase contrast photomicrographs of PMA-treated muscle cell cultures. (*A*) Control cultures treated with vehicle (0.1% DMSO) for 24 h. (*B*) Cultures treated for 24 h with PMA (75 ng/ml and 0.1% DMSO). Arrows identify nuclei which in control cultures are aligned along the longitudinal axes of the myotubes and in treated cultures are aggregated in the center of the myosacs.

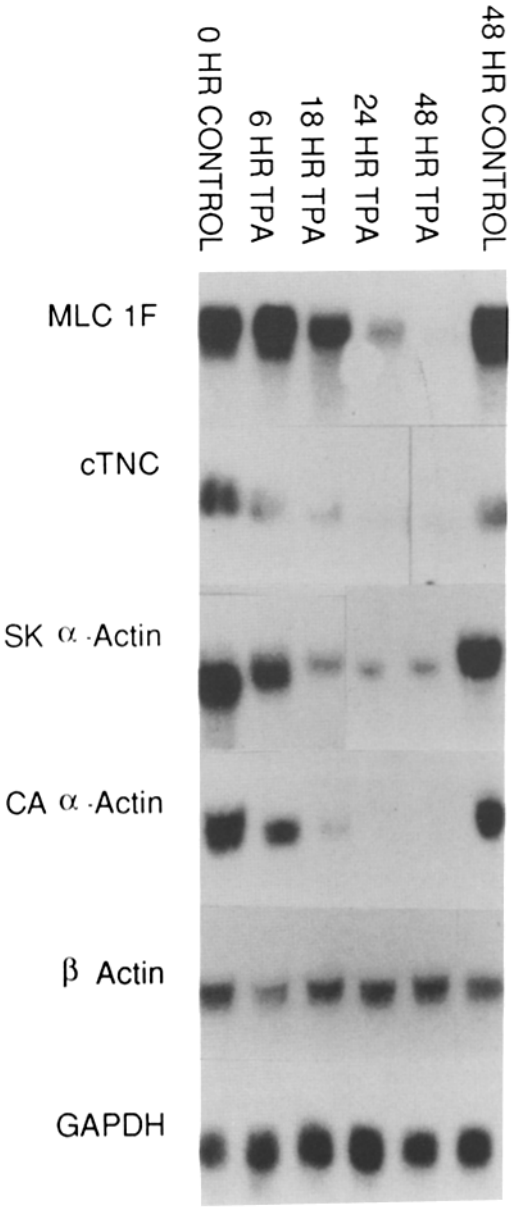

Figure 2. Loss of muscle-specific mRNAs in PMA-treated cultured muscle cells. Autoradiogram of a representative experiment in which total RNA from PMA (*TPA*)-treated cultures was hybridized with radiolabeled cDNA probes for the alkali first fast myosin light chain (*MLC 1F*), cardiac troponin C (*cTnC*), skeletal α-actin (*SK α-Actin*), a-cardiac α-actin (*CA* α-Actin), β-cytoplasmic actin (*β-Actin*), and glyceraldehyde phosphate dehydrogenase (*GAPDH*). (PMA [TPA], final concentration of 75 ng/ml and 0.01% DMSO) was added to 100–120-h cultures of ED 12 chicken pectoral muscle for the times indicated and total RNA from the cultures was then harvested as described in Materials and Methods.

nonmuscle actin (β-actin) and the constitutively expressed glycolytic enzyme, GAPDH, remain unperturbed or even slightly increased after the addition of PMA.

PMA Inhibits the Transcription of Some but Not All Skeletal Muscle-specific Genes

To examine the level of regulation at which PMA exerted its selective influence over muscle-specific mRNA accumulation, we examined its effects on the transcription of muscle-specific and nonmuscle genes. Nuclear transcription run-on assays were conducted to measure the synthesis of nascent RNA transcripts in 5-d-old muscle cultures after an 8-h treatment with PMA at 75 ng/ml. A typical dot blot from a single run-on experiment is shown in Fig. 3 *A*, while a compilation of data from at least three independent experiments are shown in Fig. 3 *B*. Hybridization of de novo synthesized ^{32}P-labeled RNA transcripts to the specific cDNA sequences indicated on the blot showed that transcriptional initiation at the time nuclear isolation of β-actin and GAPDH were not significantly blocked. The transcription of the cardiac α-actin and troponin C genest, for example, was inhibited by ~70 and 80%, respectively, while the transcriptional activity of other muscle genes (e.g., skeletal α-actin and fast MLC1f) were not significantly affected.

PMA Destabilizes Muscle Contractile Protein mRNAs

Using pulse-chase kinetic analyses, we next examined whether the changes in mRNA accumulation were accompanied by changes in mRNA stability. Cellular RNA labeled

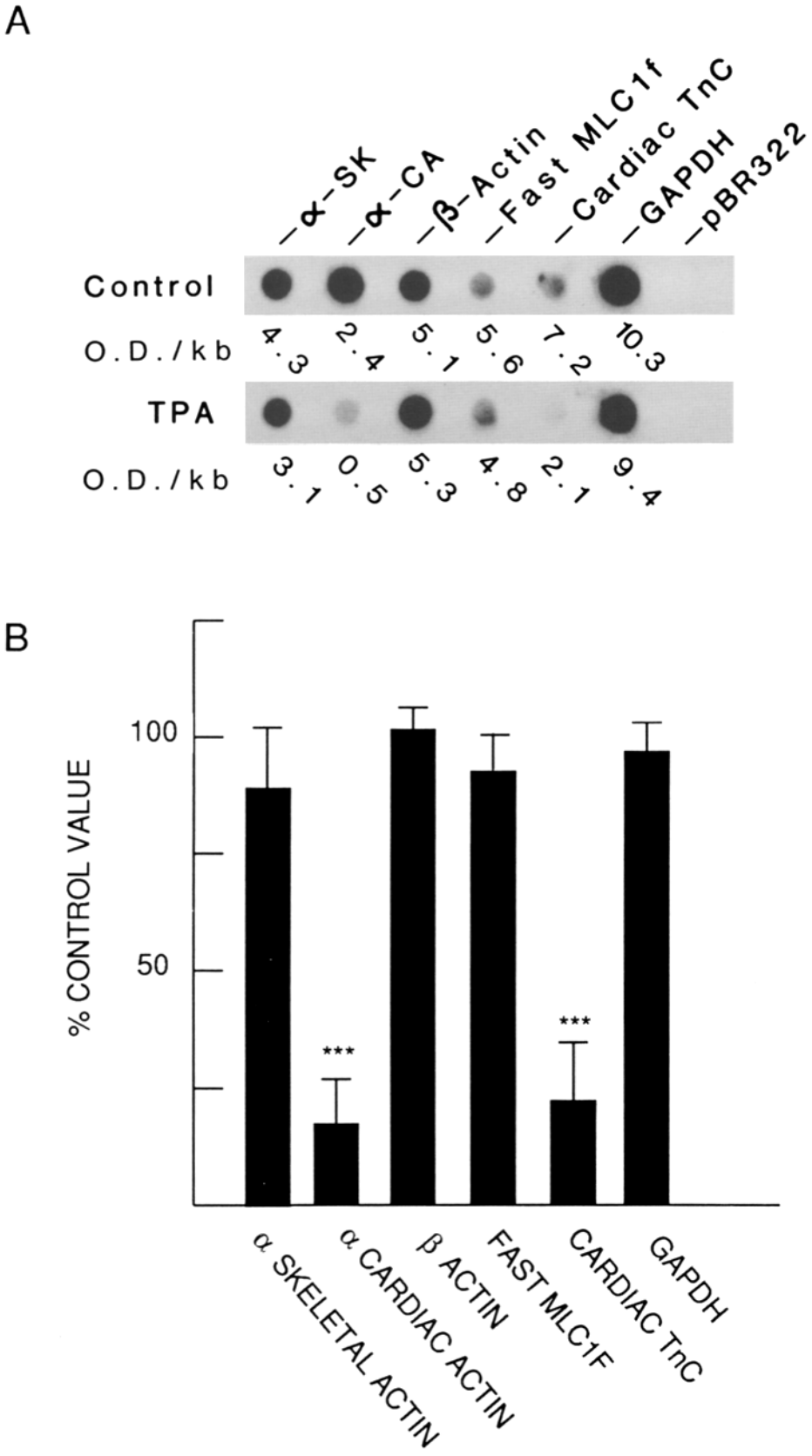

Figure 3. The effect of PMA on muscle gene transcription. (*A*) Autoradiogram of a representative dot blot of run-on transcription in nuclei isolated from control and PMA (*TPA*)-treated (75 ng/ml for 8 h) muscle cell cultures. cDNA and PCR probes corresponding to the genes examined were bound to Nylon and then hybridized with radiolabeled in vitro run-on transcripts. The blot was then treated as described in Materials and Methods. A parallel autoradiogram in which the maximum OD $A_{540} < 1.5$ was analyzed by densitometry. The number below each dot is the optical density of the radiographic image normalized to size of the probe used to detect the run-on transcripts. (*B*) Bar graph analysis of densitometric measurements from several independent experiments. The effects of PMA (*TPA*) on transcription of each gene are expressed as a percentage of control. Error bars indicate one standard deviation from the mean. (***) indicates values that are significantly different from control ($p > 0.001$).

with [^{3}H]uridine was chased with cold uridine and cytidine and the level of specific mRNAs assayed by hybridization to denatured DNA probes cross-linked to nylon filters. The kinetic data for calculating the mRNA turnover are presented in Fig. 4 and the calculated mRNA half lives in Table I. In untreated cultures, the fast MLC1f mRNA was the most stable of all the mRNAs examined with a calculated $t_{1/2}$ >60 h. Skeletal α-actin, cardiac α-actin, and cardiac troponin C had

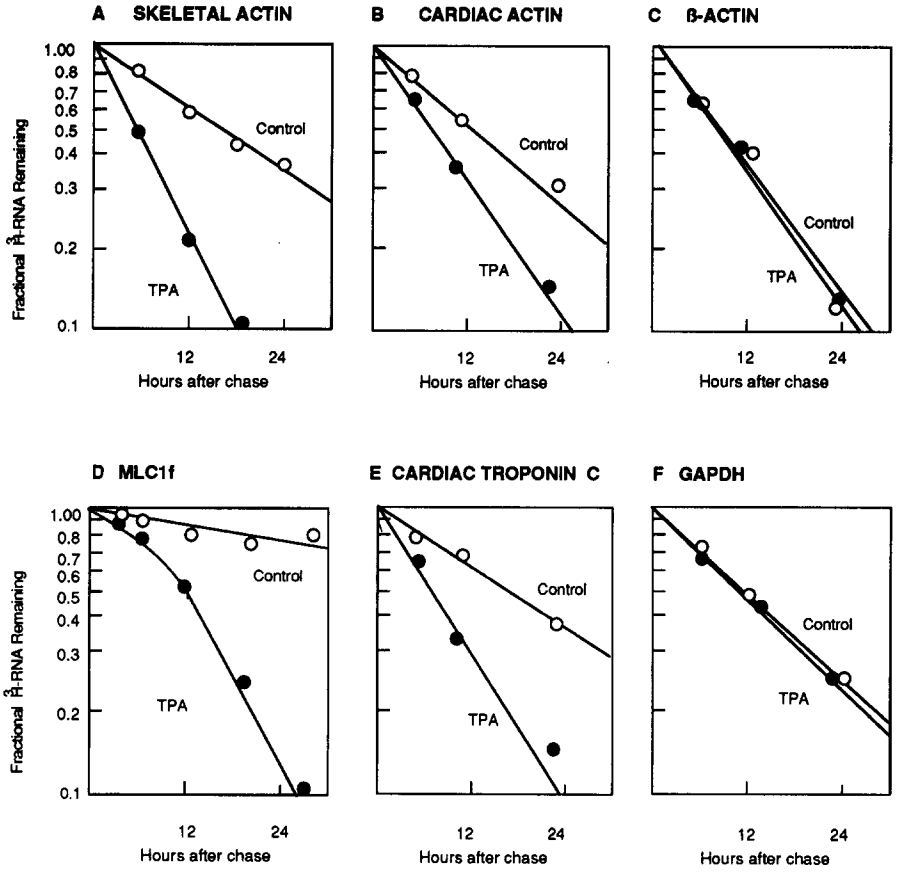

Figure 4. Analysis of mRNA turnover in PMA-treated cultures. Decay of [^{3}H]uridine-labeled mRNAs after an extended chase after the addition of PMA/DMSO (*TPA*) or DMSO alone (*control*). Total RNA was radiolabeled by incubating cultures for 48 h in [^{3}H]uridine as described in Materials and Methods. Radiolabeled uridine was then removed and the cultures "chased" for 4 hours with unlabeled uridine and cytidine. PMA or vehicle (DMSO) was then added and cultures were then harvested at the times indicated. RNA was isolated from the cultures and hybridized to cDNA and PCR probes that were immobilized on Nylon membranes. The level of specific mRNA was quantitated either by densitometry of the blots after fluorography or by scintillation counting of eluted radiolabeled RNA bound to denatured cDNA or PCR probes immobilized on Nylon. All values are expressed relative to the value at the beginning of TPA addition. All datapoints represent the mean of three independent determinations with the standard error of that mean contained within the area of the symbols.

normal mRNA half-lives of 19.4, 13.5, and 14.7 h, respectively, while the nonmuscle β-actin and GAPDH mRNA displayed relatively short half-lives of 8.4 and 10.7 h, respectively.

Compared with the normal decay of the mRNA molecules, PMA shortened the half-life of all the muscle-specific mRNAs. The most dramatic effect of PMA on mRNA turnover was observed for fast MLC1f with its half-life reduced from >60 h to ~5 h with PMA. This rate, however, was not achieved until 10–12 h after the addition of PMA. The decrease in mRNA half-lives for the sarcomeric actins and cardiac TnC, on the other hand, was achieved within 1–3 h after adding PMA. PMA reduced the half-lives of all three thin filament mRNAs to a similar level (4.9–5.7 h), but had no effect on the stability of either β-actin or GAPDH mRNAs.

Table I. Half-Lives of Muscle and Nonmuscle mRNAs in Primary Muscle Cell Cultures Treated with PMA

mRNA	Half-lives	
	Control	PMA
	h	
Skeletal α-actin	19.4 ± 2.4*	4.9 ± 0.7
Cardiac α-actin	13.5 ± 1.6	5.7 ± 0.6
β-actin	8.4 ± 1.1	8.6 ± 1.2
MLC1f	>60	4.8 ± 0.5
Cardiac TN C	14.7 ± 1.2	5.1 ± 0.4
GAPDH	10.7 ± 1.4	10.5 ± 1.7

* Values expressed are the mean ± standard deviation of the mean. At least three separate determinations were done for each measurement.

The Accelerated Turnover of mRNA in PMA-treated Myotubes Is Blocked by Transcription Inhibitors

Attempts to measure mRNA stability by monitoring total accumulation after inhibiting RNA synthesis were unsuccessful since such inhibitors appeared to actually block the decrease in mRNA accumulation that occurred after PMA addition. This effect is illustrated in Fig. 5, which shows a typical experiment in which the RNA transcription inhibitor, 5,6-dichloro-1-D-ribobenzimidazole (DRB, 60 μM), was applied alone or in combination with 75 ng/ml PMA to cultured muscle cells. Total cellular RNA isolated from myotubes during an 18-h time course was then probed for skeletal α-actin, MLC1f, and β-actin mRNA levels. As before, PMA caused an extensive decrease in both MLC1f and skeletal α-actin mRNA accumulation after 18 h of exposure. For both muscle genes, DRB resulted in an unexpected increase in mRNA levels in both control and PMA-treated cultures. This increase in PMA-treated cultures was large enough to suggest that DRB blocked the action of PMA. As before, the level of β-actin mRNA accumulation was not changed by PMA. DRB alone or in combination with PMA resulted in a significant reduction in β-actin mRNA levels, probably reflecting the decay of mRNA after DRB's inhibition of β-actin transcription.

Similar results were obtained with other transcriptional inhibitors, such as actinomycin D (10 μg/ml), although the effects of transcriptional inhibition were not as reversible as with DRB. In addition, the decrease in MLC1f levels after PMA administration was also blocked by coadministration of the translation inhibitor, cycloheximide, at doses that inhibited protein synthesis by >90% (10 μM).

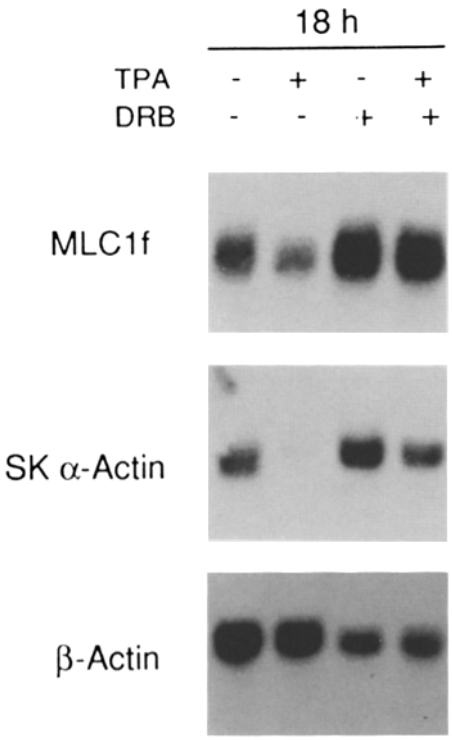

Figure 5. An RNA transcription inhibitor blocks PMA's effect on mRNA stability. Northern blot analyses of RNA from cultures treated with DRB, TPA (PMA), and DRB/TPA and hybridized with radiolabeled cDNA probes to MLC1f, skeletal α-actin, and β-actin. PMA (75 ng/ml in DMSO) and 5,6-dichloro-1-D-ribobenzimidazole (*DRB*, 5 μg/ml) were added to 100–120-h cultures of ED 12 chicken pectoral muscle alone or in combination and total RNA from the cultures was harvested after 18 h.

PKC Is Depleted in PMA-treated Cultured Chicken Muscle Cells

In many cell types, the principal mechanism of phorbol ester action is to bind to the regulatory domain of the calcium-phospholipid–dependent PKCs in the cell and to alter their activity. Such binding initially leads to PKC activation, but continued interaction results in decreased activity as PKC protein levels are downregulated (Ballester and Rosen, 1985; Hepler et al., 1988). The kinetics of this effect depends on the concentration of phorbol ester and the cell type to which it is administered. To determine what effect PMA is having on PKC content in the chicken muscle cell cultures over the time course of our experiments, we measured the relative levels of PKC in control and PMA-treated cultures by phorbol 12,13-dibutyrate (PdBu) binding. The binding characteristics of the radiolabeled phorbol were first determined by incubating untreated cells with increasing amounts of the labeled phorbol ester. Specific binding of the label was saturable at 1.56 ± 0.23 pmol/well or 2.15 ± 0.31 pmol/mg protein (Fig. 6 *A*) and a Scatchard analysis of the data (*inset*, Fig. 6 *A*) revealed a dissociation binding constant (K_d) of 36.4 ± 1.9 nM (~20 ng/ml). While this value is significantly higher than the K_ds reported for many replicating cells, including myoblasts, it is similar to that observed for differentiated mammalian muscle cells (Martelly et al., 1989).

Table II. PKC Activity in Control and PMA-treated Chicken Muscle Cell Cultures

	PKC Activities		
	(1) soluble	(2) particulate	(1 + 2) total
	μmol/min/mg protein		
T = 0			
Control	27.9 ± 2.5	59.4 ± 6.1	87.3 ± 8.1
T = 8 h			
Control	31.2 ± 3.7	61.5 ± 4.5	92.7 ± 8.4
PMA	3.5 ± 1.3	27.4 ± 1.9	30.9 ± 3.0
T = 16 h			
Control	31.7 ± 2.6	52.6 ± 4.6	82.3 ± 7.1
PMA	2.1 ± 1.2	13.4 ± 2.3	15.5 ± 3.2
T = 24 h			
Control	22.3 ± 1.8	56.8 ± 4.5	79.1 ± 5.9
PMA	1.8 ± 0.8	6.3 ± 1.2	8.1 ± 1.8
T = 48 h			
Control	23.7 ± 2.1	64.6 ± 3.6	88.3 ± 5.4
PMA	0.6 ± 0.4	2.4 ± 1.3	3.0 ± 1.4

Values expressed are the mean ± standard deviation of the mean. At least three separate determinations were performed for each measurement.

A saturating dose of the labeled PdBu was then used to obtain a relative measure of PKC levels in cells exposed to PMA for various times (Fig. 6 *B*). Significant reductions in PdBu binding signaling a downregulation of PKC levels were evident as early as 8 h after PMA addition. After 24 h, <5% of the binding activity detected in untreated parallel controls could be seen in the muscle cells. Therefore, over the period during which contractile protein mRNAs were being selectively depleted from the cells, PdBu binding to PKC was significantly reduced. If, as in other cell types, changes in PdBu binding reflect changes in the amount of intact PKC enzyme, total activatable enzyme would be reduced to such low levels that, even if it were fully active in vivo, PKC activity would be significantly reduced at the earliest times after PMA addition.

To corroborate these findings, PKC activity in both soluble and particulate cell fractions was also measured (Table II). Total activatable enzyme is presented as the sum of enzyme activity in both subcellular compartments, while estimated in vivo activity is represented by that in the particulate (membrane) fraction. The results presented in Table II show a substantial decrease in the total activatable pool of PKC in PMA-treated cultures that parallels the reduction in phorbol ester binding seen in Fig. 6 *B*.

Discussion

PMA at doses identical to those used in this study have been shown by others to cause a selective disassembly of the sarcomeric structures in primary cultures of avian embryonic muscle and the subsequent degradation of their protein constituents (Lin et al., 1987, 1989). We demonstrate here that these morphological and protein biochemical changes are also accompanied by dramatic reductions in the accumulation of mRNAs encoding the contractile proteins and that this reduction is selective for the mRNAs encoding the con-

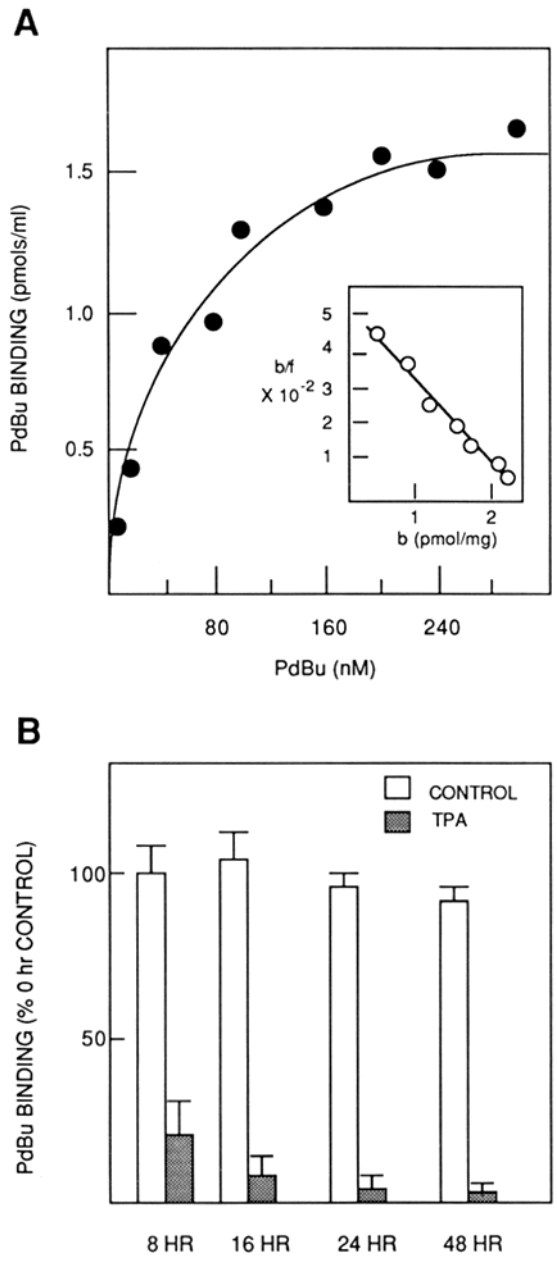

Figure 6. Analysis of phorbol binding to cultured chicken muscle cells. (*A*) Saturation curve for [^{3}H]phorbol 12,13-dibutyrate binding to cultured chicken muscle cells. Binding is expressed as picomoles bound per milliliter extract and as picomoles per milligram protein extract in the Scatchard analysis shown in the inset. Data represent three independent determinations. Standard error of the mean for each datapoint in within the area of the symbol. (*B*) Effects of preincubation of cells with PMA for the times indicated on saturable phorbol dibutyrate binding. Cells were incubated with 200 nM [^{3}H]PdBu and specific binding determined as described in Materials and Methods. The values for treated and parallel control cultures are expressed relative to the binding values determined at the zero timepoint for the experiment.

tractile proteins. The steady-state levels of different mRNAs reflect the balance between the rate of synthesis of new mRNAs and the rate of mRNA degradation. Pulse-chase kinetic analyses indicated that PMA treatment of differentiated muscle cells resulted in a dramatic destabilization of all the contractile protein mRNAs examined. Nuclear run-on assays indicated that expression of the cardiac α-actin and troponin C genes was also inhibited at the transcriptional level by 70–80%, while skeletal α-actin, fast MLC1f, and the nonmuscle β-actin and GAPDH genes were not significantly effected. It has previously been shown that the cardiac and skeletal α-actin genes are expressed in a sequential manner during muscle development (Hayward and Schwartz, 1986) and that skeletal α-actin but not cardiac α-actin gene expression can be selectively blocked by reducing extracellular calcium (Hayward et al., 1988). The results presented here on the differential effect of PMA on transcription of these isoforms reveals another fundamental difference in the way these sarcomeric α-actin genes are regulated.

The changes observed in mRNA accumulation upon PMA addition (Fig. 2) are consistent with the effects of this drug on the transcription of the muscle genes and the stability of their mRNA products. In the case of both cardiac α-actin and troponin C, mRNA levels fell the quickest and remained the lowest, an outcome predicted by the fact that transcription of these genes was virtually shut down by PMA and that the stability of the existing mRNA markedly decreased. The transcription of the skeletal α-actin and MLC1f genes, on the other hand, were relatively unaffected by PMA. The mRNA content of these genes fell to levels of accumulation of ~25% for skeletal α-actin genes and 5–10% for MLC1f, these changes reflecting solely the reduction in mRNA half-life by PMA. Poly A$^+$ RNAs from muscle cell cultures were previously shown to be distributed into two populations with regard to their stability. Medford et al. (1983) showed that one RNA population had a $t_{½}$ <4 h and the other a $t_{½}$ which ranged from 17 to >54 h, with muscle-specific proteins apparently encoded by the longer-lived mRNAs (Buckingham et al., 1976; Medford et al., 1983). We found that the $t_{½}$ of primary muscle cell culture mRNAs was partitioned in a similar manner, in which the muscle-specific mRNAs were the most stable and the nonmuscle mRNAs among the least stable (Table I). PMA dramatically reduced the stability of all the muscle-specific mRNAs tested to level that was similar to that of the nonmuscle-specific mRNAs (e.g., ~5 h). The stability of the nonmyogenic mRNAs remain unchanged by PMA.

The method used to estimate the stability of specific mRNAs relied on labeling the mRNA pool and then following the loss of label from that pool. This method may fail to give a truly accurate measurement of degradation rates because the specific activity of the labeled mRNA pool is continually being reduced as newly synthesized mRNA transcripts are added to the pool. For most of the mRNAs studied, however, this method gives a reasonable measurement of the relative differences between the control and treated states, because the error contributed by transcription is the same for both control and treated cultures. But since the transcription of both the cardiac α-actin and cardiac troponin C genes are significantly reduced by PMA, this method underestimates the relative difference between the degradation rates in control and treated cultures, since PMA inhibits the transcription of these genes. If for the sake of argument, we imagine that PMA had no effect on the stability of either cardiac actin or troponin C mRNAs, then at any given time after labeling and during the chase, the specific activity of label in the mRNA pool of control cultures will

be less than that in the PMA-treated cultures because of the differential contribution of newly synthesized transcripts in the control cultures. Over time, the specific activity of the control culture would decrease and it would appear that PMA decreased the rate of degradation and stabilized the mRNA. To compare the decay rates in treated and untreated cultures, an adjustment must be applied to the data to compensate for this artifact. In the case of the mRNAs for cardiac actin and troponin C, the difference in decay rates between control and PMA-treated cultures and the effect of PMA on destabilizing these mRNAs is even greater than we measure. While such adjustments were not applied to the data shown in Fig. 4 and Table I, they would only serve to strengthen the conclusions concerning the destabilization of these cardiac isoform mRNAs relative to control values and not to negate or weaken them.

The selective turnover of the striated α-actin mRNAs versus the nonmuscle β-actin shows for the first time that sequence differences between these evolutionarily conserved actin mRNA isoforms may have a role in determining their intrinsic half-lifes and cellular content. Comparison of the untranslated regions in vertebrate skeletal α-, cardiac α-, and β-actin mRNA has revealed regions of high sequence homology within the 3′ portion of each of these actin isoformic mRNAs and that this homology is greater among the α-striated isoforms than between the α- and β-isoforms (Mayer et al., 1984; Ponte et al., 1984; Chang et al., 1985). In comparison, other vertebrate genes such as those encoding insulin (Lomedico, 1979) and prolactin (Cooke et al., 1981), which also share common coding regions usually contain divergent 3′ untranslated regions. The preservation of the 3′ untranslated regions for over 300 million years of evolution suggests that they have important biological roles for the expression of the actin genes and the results presented here implicate these at the level of mRNA stability.

Since transcription inhibitors blocked the PMA-induced changes in muscle-specific mRNA turnover (Fig. 5), the induction of de novo transcripts by PMA may play an important role in the accelerated turnover of these mRNAs. The fact that the protein synthesis inhibitor, cycloheximide, also prevented the PMA-induced destabilization of muscle-specific mRNAs (unpublished observation) suggests that the transcripts are translated into proteins with relatively short half-lifes. A similar requirement for protein synthesis to observe the other effects of PMA on differentiative properties in muscle has been described previously (Cossu et al., 1982). These proteins could be directly involved in the destabilization process as has been shown for *c-myc*, in which a labile protein destabilizer with an essential nucleic acid component was recently identified (Brewer and Ross, 1989). On the other hand, it is possible that all these inhibitors alter the ability of the cells to respond to PMA. However, cells treated with these inhibitors and then exposed to PMA still exhibit many of the morphological changes that are visible at the light microscopic level and that occur in untreated cells in response to PMA (unpublished observations). These include the deposition of muscle nuclei into a centralized region of the myotube and the loss of elongated structure to form a myosac. Experiments are underway to determine if the immunochemical changes that have been described in PMA treated cells also occur in DRB and CHD-treated cells in response to PMA.

The primary mode of phorbol ester action in many experimental systems is to bind to and alter PKC activity. Critical to the interpretation of the results presented here is what effect PMA may have on PKC level and activity throughout the period of sarcomere disassembly and message loss. While PMA has activated PKC, it can provide a dual effect on PKC activity, causing first a short-term activation and then an inhibition as a result of the degradation of the molecule (Ballester and Rosen, 1985; Hepler et al., 1988). At the doses used in this study and the time at which its effects were assayed, PMA is acting to inhibit PKC levels and activity (Fig. 6 and Table II). Consistent with the fact that the effects we have observed are correlated with PKC inhibition and not activation is that treatment of the cells with known PKC activators, such as the permeable diacylglycerol analogues oleoyl-acetyl-sn-glycerol and dioctanoylglycerol, slightly elevated muscle specific mRNAs rather than decreasing them, while inhibitors of protein kinase C, such as staurosporine and H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride], decreased expression of skeletal α-actin mRNA, while not affecting β-actin expression (data not shown). While both staurosporine and H7 can have inhibitory effects on other protein kinases (Tamaoki et al., 1986; Love et al., 1989), the common specificity of these drugs toward PKC suggests that the observed effects on contractile protein mRNA content might be, in part, due to PKC inhibition.

PMA-induced disassembly of striated myofibrils was shown by Holtzer and collaborators (Lin et al., 1987, 1989) to be a multistep process in which I-Z-I complexes containing α-sarcomeric actins (I) and α-actinin (Z) were selectively released from the myofibrils within 5–10 h to form 3 μM cortical actin-containing bodies (CABs). The myosin-containing A bands were then disrupted only after 15–20 h in PMA. At the protein level, immunologically detected cellular α-actin was lost after 30 h of treatment, while MHC-staining amorphous patches were evident up to 72 h. Our data on the temporal pattern of muscle specific contractile mRNAs reduction displays a striking consistency with this temporal order of sarcomere disassembly. The mRNAs encoding α-cardiac actin, α skeletal actin, and cardiac troponin C, all thin filament (I band) proteins, are significantly reduced after only 5 h of treatment. MLC1f, a thick filament (A band) component, on the other hand, declined at a slower rate consistent with the slower disappearance of A-band associated proteins. Kinetic analysis of this disappearance identifies two components to the apparent slower rate of loss: (*a*) a delay of ~10 h after PMA addition during which MLC1f mRNA decayed at a nonlinear rate, followed by (*b*) a linear rate of decay similar to that of the mRNA for the thin filament proteins. This delay in degradation of the MLC1f mRNA is consistent with the hypothesis that the destabilization of the contractile protein mRNAs is an event secondary to the loss of sarcomeric structure and the degradation of the contractile apparatus rather than a consequence of a separate intracellular catabolic pathway activated by phorbol esters. If the stability of the contractile proteins were, in turn, dependent on their assembly into contractile structures, the stability of contractile protein mRNAs would then be determined by the stability of their encoded gene products.

Finally, the selective transcriptional effect of PMA on cardiac α-actin and troponin C genes is particularly intrigu-

ing in light of the regulation of these genes in ovo. The cardiac and skeletal α-actin genes are expressed in a specific manner during muscle development (Hayward and Schwartz, 1986) with the cardiac isoform being the predominant species expressed in early embryonic development of the chick. The transition from early to late embryonic expression is then marked by the downregulation of cardiac-type isoforms and the upregulation of their skeletal-type counterparts. The response of cultured muscle cells to PMA mimics one part of the molecular events associated with this developmental transition (i.e., the downregulation of early embryonic cardiac-type isoform expression) and implicate changes in PKC as a possible intracellular messenger linking extracellular signals and developmental gene expression (Papadopoulos and Crow, 1991). Careful studies of how cells respond to changes in PKC and particular PKC isozymes may provide insight into how muscle cells regulate isoform-specific expression from gene families during development and in various physiological and pathophysiological circumstances.

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