Effects of Phorbol 12-Myristate 13-Acetate on the Differentiation Program of Embryonic Chick Skeletal Myoblasts

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ABSTRACT

The effects of phorbol 12-myristate 13-acetate (PMA) on three aspects of myogenesis have been analyzed: (a) fusion of mononucleated myogenic cells to form myotubes; (b) synthesis and accumulation of two muscle-specific proteins; and (c) DNA synthesis. Using autoradiography combined with immunofluorescent localization of muscle-specific light meromyosin and the muscle-specific intermediate filament protein desmin, we have found that embryonic chick myogenic cells cultured in the presence of PMA (50 nm) initiate the synthesis of both desmin and muscle-specific light meromyosin and, by these criteria, partially differentiate. These cells differ from normal definitive postmitotic myoblasts, however, since they (a) do not fuse; (b) do not assemble normal myofibrils; and (c) incorporate [3H]thymidine. PMA does not appear to induce DNA synthesis in postmitotic myoblasts, but it apparently permits cells to initiate expression of muscle-specific proteins while preventing complete withdrawal from the cell cycle. Inhibition of fusion by PMA has been reported, but continued incorporation of [3H]thymidine in nuclei of cells expressing muscle-specific proteins is a previously undescribed effect of PMA. This effect is not achieved by 4-α-phorbo1-12,13-didecanoate, a nonpromoting phorbol ester, and may be relevant to the action of PMA as a tumor promoter.

INTRODUCTION

The tumor promoter PMA6 has a wide variety of effects, including either the inhibition or stimulation of cell differentiation (for reviews, see Refs. 12, 28, 52, and 54). With respect to differentiation, the effect of PMA differs depending on cell type (13, 34, 43), species (21, 41, 45, 46), and in what compartment of its lineage the cell is (11, 35, 44). One of the first systems in which PMA was shown to reversibly inhibit different aspects of differentiation was in vitro myogenesis (8–10). PMA has been shown to (a) reversibly block the fusion of postmitotic myoblasts into multinucleated myotubes, (b) degrade striated myofibrils into amino acids, and (c) inhibit the striking increase in total Ca2+ which occurs as normal myotubes mature. Yeoh and Bright (55) reported that, while PMA inhibited fusion, the cultures did accumulate small amounts of muscle-specific M-CK. However, this study did not determine whether the M-CK was synthesized by the definitive postmitotic myoblasts present at the time of plating or by the progeny of cells that replicated in vitro in the presence of PMA.

Using [3H]dThd autoradiography combined with immunofluorescent localization of light meromyosin and desmin, we have analyzed the effects of PMA on 3 aspects of myogenesis: (a) fusion into myotubes; (b) synthesis of muscle-specific proteins; and (c) synthesis of DNA. We have found that myogenic cells cultured in PMA initiate the synthesis of both desmin and light meromyosin and, by these criteria, differentiate partially. However, these cells differ considerably from normal definitive postmitotic myoblasts in that (a) they do not fuse to form myotubes; (b) they fail to assemble striated myofibrils; and (c) they incorporate [3H]dThd, and some enter mitosis. The fate of these atypical PMA-treated myogenic cells is unknown.

MATERIALS AND METHODS

Cell Cultures. Myogenic cultures were established as described previously (5) but with minor modifications. Briefly, breast muscle from 12-day-old chick embryos was dissociated in Ca2+ and Mg2+-free salt solution [CMF − 0.137 mM NaCl-2.7 mM KCl:1.5 mM Na3PO4:6 mM NaHCO3:5.5 mM glucose (pH 7.4)] containing 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.). Following centrifugation and resuspension in standard culture medium, the cells were filtered through lens tissue, and a dilution was counted in a hemocytometer. Cells were plated at a density of 3 × 105 cells/1.5 ml/35-mm tissue culture dish which was previously coated with rat tail collagen. Cells were also plated in 100-mm tissue culture dishes to be used for subculturing. Culture medium consisted of Eagle’s minimum essential medium containing 10% horse serum, 3% chick embryo extract, 0.2 mM glucose, penicillin (50 units/ml), streptomycin (50 μg/ml), and Fungizone (2.5 μg/ml) (all obtained from Grand Island Biological Co., except embryo extract). The medium in all cultures was replaced daily. Cells were subcultured using a 1:10 dilution of trypsin:EDTA (final concentrations: trypsin, 0.05% and EDTA, 0.02%; Grand Island Biological Co.) in CMF for 2 to 4 min at 37°.

PMA was obtained from Dr. Peter Borchert (Eden Prairie, Minn.). Stock PMA was prepared by dissolving the agent in absolute ethanol and obtaining an absorption spectrum to calculate the concentration of phorbol compound in solution (we are grateful to Dr. T. G. O’Brien, Wistar Institute, Philadelphia, Pa., for providing the equipment and assistance needed for this procedure). PMA stock was stored in the dark at −20°, and fresh PMA medium at a concentration of 50 μM (30 ng/ml) was prepared daily. Nonpromoting phorbol ester 4-α-PDD stock solution was a gift from Dr. T. G. O’Brien. EGTA was obtained from Sigma Chemical Co. (St. Louis, Mo.), and a 40 μM stock was prepared as described (40) with minor modifications. BrdUrd (Sigma) stock was prepared by dissolving the analogue in dimethyl sulfoxide to give a 20-μg/ml solution. This was diluted 1:20 with a balanced salt solution (0.137 mM NaCl:2.7 mM KCl:1.5 mM MgCl2:1.5 mM NaN3PO4:6 mM NaHCO3:5.5 mM glucose, pH 7.4) to give a 1-μg/ml working stock which was passed through a 0.22-μm filter and stored frozen at −20°.

Antibodies. Preparation and characterization of antibodies against light meromyosin and desmin have been described (3, 10, 16, 17). In...
cultures derived from embryonic chick skeletal muscle, both of these proteins are present in definitive postmitotic mononucleated myoblasts and myotubes and are thus markers of cells in the terminal compartment of the myogenic lineage (23, 24, 29).

Immunofluorescent Microscopy. Cells were processed for immunofluorescent microscopy as described elsewhere (49) with only minor modifications. The DNA-binding fluorochrome, bisbenzimide (Hoechst No. 33258 (49)), was used as a nuclear counterstain. Cells were examined using either Zeiss or Leitz epifluorescent microscopes with appropriate filters for either fluorescein;bisbenzimide or rhodamine fluorescence.

Immunofluorescent Autoradiography. Autoradiography was combined with the immunofluorescent technique to determine if cells binding muscle-specific antibody incorporated \(^{3} \text{H}\)dTdr into their DNA. Cultures were established as described above but at a density of 5 \times 10^6 cells/1.5 ml/dish. Cells were exposed to \(^{3} \text{H}\)dTdr (0.1 to 0.5 \(\mu \text{Ci/ml}\)) (specific activity, 50 Ci/mmol; New England Nuclear, Boston, Mass.) for varying periods of time as indicated for each experiment. Cultures which were not sacrificed immediately were maintained in medium containing 0.32 mm unlabeled dThd to prevent reuse of isotope (6). After immunofluorescent processing, the cultures were coated with a 1:1 dilution of NTB2 emulsion (Kodak, Rochester, N. Y.) at 45\(^\circ\) and air-dried for 30 to 90 min. Cultures were stored in light-tight boxes and exposed for approximately 1 day at 4\(^\circ\) and for the remaining 1 to 6 days at either 4\(^\circ\) or -70\(^\circ\). Autoradiographs were developed using D-19 developer (Kodak) for 2 min, rinsed with deionized water, fixed, washed for 5 to 15 min with deionized water, and mounted as described above. A cell with greater than 30 to 40 grains overlying its nucleus was scored as labeled positively; frequently, most of the nucleus was obscured by grains.

RESULTS

Fusion. In control cultures, there was extensive fusion of mononucleated cells into myotubes (Fig. 1, a and b); in PMA cultures, fusion was inhibited (Fig. 1, e and f). However, there was a small number (\(\text{circa} 1 \text{ to} 2\%\)) of nuclei in Day 2 primary PMA cultures within oligonucleated cells (Fig. 5). Typically, these structures contained 2 to 12 nuclei and were both desmin and light meromyosin positive, although these proteins were not arranged in recognizable striated myofibrils. It is possible that these small, atypical myotubes originated from mononucleated myoblasts that had fused in vivo and were part of the initial inoculum. The observation that early control cultures contain a small number of oligonucleated cells (6, 7, 14) supports this interpretation.

Desmin and Light Meromyosin Expression. In contrast to the inhibition of fusion, PMA did not block the expression of muscle-specific proteins in mononucleated myogenic cells. Primary myogenic cultures were established in either control or PMA medium. Two days after plating, the cultures were fixed for immunofluorescent localization of desmin and light meromyosin. There were similar numbers of nuclei in desmin-positive cells in the control (73 nuclei/sq mm) and the PMA (69 nuclei/sq mm) cultures. In control cultures many of these nuclei were in myotubes, whereas in PMA-treated cultures greater than 96% of the nuclei were in mononucleated cells, with the remainder in the oligonucleated cells described above. Similarly, the number of nuclei in light meromyosin-containing cells in PMA cultures was comparable to that in control cultures. Double staining with antibodies to both desmin and light meromyosin showed that the light meromyosin-positive cells were always desmin positive, whereas many desmin-positive cells were light meromyosin negative.

Cultures were stained with antibodies to both desmin and light meromyosin at various times after plating. In these cultures, the desmin-positive population increased several-fold with time in both control and PMA cultures (Fig. 1). Polyacrylamide gel electrophoresis of insoluble proteins demonstrated a band corresponding to desmin in both control and PMA cultures on Day 6. Immunolabeling of this protein after transfer to nitrocellulose confirmed its immunological identity to desmin (data not shown). Similar to desmin expression, the number of cells containing light meromyosin increased during the first 6 days in culture. By Day 10, however, PMA cultures double-stained using both antibodies contained many cells which fluoresced brightly with anti-desmin but often bound little anti-light meromyosin. This loss of light meromyosin staining may be related to the selective degradation of light meromyosin in myotubes exposed to PMA (9, 10). Because of the diminution of light meromyosin with culture in PMA medium, this study relies mainly on the synthesis of desmin and to a lesser extent on the synthesis of light meromyosin as markers of myogenic differentiation.

The expansion of the desmin-positive cell population in the presence of PMA as seen in Fig. 1 suggests either the initiation of myogenic differentiation of cells "born" in culture or replication of the cells that were desmin positive at the time of plating. In order to test the first possibility, we used BrdUrd to obtain myogenic cultures containing very few desmin- and light meromyosin-positive cells. BrdUrd has been shown to inhibit myogenic differentiation (7), and cultures maintained in BrdUrd consist almost entirely of replicating presumptive myoblasts and fibroblasts. We established primary myogenic cultures in BrdUrd (10 \(\mu \text{g/ml}\)) at a density of 5 \times 10^5 cells/35-mm dish and, after 4 days \textit{in vitro}, the cells were subcultured into either BrdUrd medium, PMA medium, or control medium. As shown in Fig. 2, the great majority of cells maintained in secondary BrdUrd cultures for up to 6 days neither fused nor initiated the synthesis of desmin. In contrast, secondary cultures grown in control medium displayed large numbers of cross-striated, multinucleated myotubes. One hundred \% of these myotubes bound anti-desmin to longitudinal filaments of indeterminate length (Fig. 2g). Six days after subculture into PMA medium, many cells had initiated desmin synthesis (Fig. 2k). Similar results were obtained with light meromyosin staining. These experiments demonstrate that, while PMA inhibits fusion in myogenic cultures, cells grown in PMA differentiate partially, since they initiate the synthesis of both desmin and light meromyosin. Note that the indirect immunofluorescence technique used in this study is considerably more sensitive than is the immunodiffusion analysis reported by Cohen et al. (8), which failed to demonstrate muscle-specific myosin in PMA-treated cultures.

Cell Cycle. To test the effects of PMA on withdrawal from the cell cycle, primary myogenic cultures, 36 hr after plating into either control or PMA medium, were pulsed for 45 min with \(^{3} \text{H}\)dTdr (0.5 \(\mu \text{Ci/ml}\)) and immediately fixed and processed for immunofluorescent localization of desmin and light meromyosin and autoradiography. The percentages of the total cell populations labeled with \(^{3} \text{H}\)dTdr were roughly similar in the control and PMA cultures, 35 and 45\%, respectively. In contrast to the total cell population, in the control cultures, only about 1\% of the nuclei in desmin-positive cells and much less than 1\% of the nuclei in light meromyosin-positive cells incorporated \(^{3} \text{H}\)dTdr, reflecting the postmitotic status of the majority of these cells. In PMA cultures, however, many of the nuclei in desmin- and light
meromyosin-positive cells incorporated \(^{3}\text{H}\text{dThd}\) (Fig. 3). In these cultures, the labeling indices of the desmin- and light meromyosin-positive cells were close to that of the total cell population (desmin positive, 37%; light meromyosin positive, 22%). Labeling similar cultures for 24 hr with \(^{3}\text{H}\text{dThd}\) (0.1 \(\mu\text{Ci/ml}\)) showed that only 13% of the nuclei in desmin-positive cells did not incorporate label and were therefore potentially postmitotic myoblasts at the time of labeling, as compared to 53% in the control cultures. These experiments indicate that, in the presence of PMA, most of the desmin- and light meromyosin-positive cells incorporated \(^{3}\text{H}\text{dThd}\) into their DNA. Furthermore, occasional observations of desmin- and light meromyosin-positive cells in various stages of mitosis confirmed that at least some of these cells began karyokinesis and were not simply involved in a high rate of reparative DNA synthesis (Fig. 4). It is noteworthy that 4-\(\alpha\)-PDD, a nonpromoting phorbol ester, did not produce continued \(^{3}\text{H}\text{dThd}\) uptake in desmin-positive cells under conditions identical to those described above (data not shown), suggesting that this effect of PMA may be related to its tumor-promoting ability.

It is noteworthy that nuclei within the atypical oligonucleated myotubes seen in early primary PMA cultures occasionally incorporated \(^{3}\text{H}\text{dThd}\). When this was observed, every nucleus within a given myotube exhibited a similar labeling density (Fig. 5). A similar type of \(^{3}\text{H}\text{dThd}\) incorporation into all of the postmitotic nuclei within injured myotubes has been commented on and probably reflects DNA repair or nonspecific uptake into degenerating nuclei (27). Mitotic figures were never seen in oligonucleated cells.

The next set of experiments was designed to examine the fate of the desmin- and light meromyosin-positive mononucleated cells that incorporated \(^{3}\text{H}\text{dThd}\) in PMA cultures. In the first experiment, we wanted to determine if, in the continuous presence of PMA, the desmin-positive cell population could be maintained through serial passages in vitro, similar to a transformed cell line (53). To this end, we subcultured myogenic cells repeatedly in either PMA or control medium and determined the number of nuclei in desmin-positive cells on the second day of each passage (Chart 1). In control medium, the number of postmitotic, desmin-positive myoblasts and myotubes declined sharply after the second subculture. This decline was thought to reflect (a) a depletion of the progenitor presumptive myoblast population that eventually produces the definitive postmitotic myoblasts; and (b) the loss of myotubes with each subculture (56). Cultures maintained in PMA medium showed a similar decline in the number of desmin-positive cells with serial passage (Chart 1). In addition, when fourth- and fifth-passage PMA cultures were subcultured into control medium, there was no increase in the number of desmin-positive cells, demonstrating that occult myoblasts were not being maintained in PMA cultures. Since the desmin-positive cell population cannot be maintained through serial passages, this experiment does not support the hypothesis of a continuously replicating desmin-positive cell population in PMA cultures.

A second experiment was designed to follow cells and their progeny for a 24-hr period. Primary cultures, 36 hr after plating into either control or PMA medium, were pulsed for 45 min with \(^{3}\text{H}\text{dThd}\) (0.5 \(\mu\text{Ci/ml}\)) and sacrificed immediately and at intervals every 4 hr. As seen in Chart 2, at Time 0 hr, few of the \(^{3}\text{H}\text{dThd}\)-labeled cells in the control culture contained desmin. During the next 12 hr, the number of \(^{3}\text{H}\text{dThd}\)-labeled nuclei in desmin-positive myoblasts and myotubes declined sharply after the second subculture. This decline was thought to reflect (a) a depletion of the progenitor presumptive myoblast population that eventually produces the definitive postmitotic myoblasts; and (b) the loss of myotubes with each subculture (56). Cultures maintained in PMA medium showed a similar decline in the number of desmin-positive cells with serial passage (Chart 1). In addition, when fourth- and fifth-passage PMA cultures were subcultured into control medium, there was no increase in the number of desmin-positive cells, demonstrating that occult myoblasts were not being maintained in PMA cultures. Since the desmin-positive cell population cannot be maintained through serial passages, this experiment does not support the hypothesis of a continuously replicating desmin-positive cell population in PMA cultures.

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positive cells in the control culture increased steadily and reached a plateau of about 17.2 nuclei/sq mm. During the next 8 hr, this number approximately doubled, presumably representing the production of new myoblasts by a second generation of presumptive myoblasts. Light meromyosin appeared significantly later than desmin in these cells and was not detectable until 12 hr after [3H]dTd labeling. The PMA-treated cultures differ from the controls in 2 major respects: (a) in the culture sacrificed at Time 0 hr, there were many labeled nuclei in desmin- and light meromyosin-positive cells; and (b) during the first 12 hr, there was an initial 50% increase in the number of labeled nuclei in desmin-positive cells followed by a slight decline; during the second 12 hr, there was another increase of about 60%. If all the desmin-positive cells that incorporated [3H]dTd at Time 0 hr proceeded to divide and yield viable desmin-positive progeny and the presumptive myoblasts produced new desmin-positive cells, the number of labeled desmin-positive cells should have increased by considerably greater than 50% with each cell cycle. Therefore, the modest 50 to 60% increase in desmin-positive cells with each cell cycle indicates that, while desmin-positive cells incorporate [3H]dTd, most do not produce viable, replicating, desmin-positive progeny in the presence of PMA.

**PMA and Postmitotic Cells.** In order to produce desmin- and light meromyosin-positive cells that incorporate [3H]dTd, PMA either could be acting on a replicating cell to block its withdrawal from the cell cycle or it could be inducing previously postmitotic cells to reinitiate DNA synthesis. To determine whether PMA could induce previously postmitotic cells to reinitiate DNA synthesis, we examined [3H]dTd uptake in both multinucleated myotubes and mononucleated definitive myoblasts in PMA and control media. In the first experiment, a 3-day-old control muscle culture containing numerous multinucleated myotubes was obtained by growing myogenic cells grown in PMA medium for an additional 24 hr. This culture and a 4-day-old control culture were then pulsed with [3H]dTd (0.5 μCi/ml) for 1 hr and sacrificed immediately for immunofluorescence and autoradiography. In both control and PMA cultures, none of the more than 5000 myotube nuclei examined incorporated [3H]dTd, which indicated that under these conditions, PMA will not induce postmitotic nuclei in myotubes to reinitiate DNA synthesis. Elsewhere, we have described the effects of PMA on myotube ultrastructure and morphology (9, 10). In a second experiment, a population enriched for postmitotic mononucleated myoblasts was obtained by growing myogenic cells in medium containing 1.75 mM EGTA (14). After 4 days in EGTA medium, the cultures were maintained for an additional 48 hr in either control medium, PMA medium, or EGTA medium; all contained [3H]dTd (0.1 μCi/ml). The results are shown in Table 1. In control medium, most of the cells had fused to form multinucleated myotubes, whereas in both EGTA and PMA cultures, cells remained mononucleated. Greater than 95% of the desmin-positive cells in both control and EGTA cultures did not incorporate [3H]dTd during the 48-hr labeling period. In PMA medium, 78% of the desmin-positive cells remained unlabeled. In all groups, the small percentage of labeled desmin-positive cells could easily have resulted from the labeling of presumptive myoblasts that produced desmin-positive progeny during the 48-hr labeling period. In conclusion, these 2 experiments do not support the hypothesis that PMA can induce detectable numbers of postmitotic myogenic cells to reinitiate DNA synthesis.

**DISCUSSION**

Our results are largely in agreement with previous studies on PMA and myogenic cells and extend their observations. As reported earlier (8, 22, 55), cells maintained in PMA did not fuse to form myotubes even after prolonged periods in culture or after multiple passages in PMA medium.

Although fusion is blocked by PMA, there is a partial activation of the terminal myogenic program. This was demonstrated by the emergence of cells that initiated the expression of muscle-specific proteins. While anti-desmin binding remained strong throughout the culture period, there was a gradual decline in the amount of detectable light meromyosin within positive cells with time. These and earlier findings suggest a loose coupling between the regulation of the synthesis of myosin heavy chains on the one hand, and the synthesis of desmin and M-CK on the other, in terminal myogenic cells (9, 10, 55). It will be of interest to determine if these atypical PMA-treated myogenic cells initiate the synthesis of other muscle-specific contractile proteins such as α-actin, the troponins, etc. (24, 30).

Probably the most provocative aspect of this study is the relationship between the initiation of the synthesis of muscle-specific proteins and DNA synthesis. In control cultures, as stressed elsewhere (1, 23, 24), the vast majority of cells initiated the synthesis of desmin and light meromyosin following their terminal S phase. Desmin appeared first, within 4 hr after the terminal S phase, and light meromyosin appeared considerably later, about 12 hr after the S phase (Chart 2). The expression of these muscle-specific proteins in control cultures was restricted almost entirely to postmitotic cells as shown by the failure of the vast majority of these cells to incorporate [3H]dTd irrespective of their ability to fuse (Chart 2, Table 1). Nonetheless, in the control cultures, a small number of nuclei (less than 1%) within desmin-positive cells incorporated [3H]dTd following a 45-min pulse and, very rarely (much less than 1%), a light meromyosin-positive cell incorporated [3H]dTd. In contrast, in the PMA cultures, a large percentage of the desmin- and light meromyosin-positive cells incorporated [3H]dTd during a 45-min pulse (Chart 2), and occasional cells in various stages of mitosis were desmin and light meromyosin positive (Fig. 4). In the present study, desmin- or light meromyosin-positive cells in mitosis were observed rarely in control cultures. Preliminary experiments in which cultures were exposed to Colcemid for 4 hr, however, indicate that a significant number of metaphase-arrested cells (circa 5%) do bind anti-desmin in control cultures. This may be the result of continued accumulation of desmin which may be first synthesized during G2 following the terminal S phase (Chart 2), making myoblasts similar to certain neuronal precursor cells.

**Table 1**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total nuclei in desmin-positive cells/sq mm</th>
<th>[3H]dTd-labeled nuclei in desmin-positive cells/sq mm</th>
<th>% of postmitotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.7 ± 18.38</td>
<td>1.1 ± 0.48</td>
<td>98</td>
</tr>
<tr>
<td>EGTA</td>
<td>27.5 ± 8.19</td>
<td>1.2 ± 0.72</td>
<td>96</td>
</tr>
<tr>
<td>PMA</td>
<td>18.8 ± 1.7</td>
<td>4.2 ± 0.64</td>
<td>78</td>
</tr>
</tbody>
</table>

*Mean ± S.E. for cell counts obtained from 3 dishes for each medium group; 5 randomly selected fields were counted per dish.*

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that initiate neurofilament expression in G₂ prior to their terminal mitosis (48).

Regrettably, our data are ambiguous regarding the fundamental issue of which type of cell in the myogenic lineage is desmin and light meromyosin positive and is induced by PMA to incorporate [³H]dThd. PMA might be altering the normal differentiation program of replicating presumptive myoblasts, allowing this type of myogenic cell to switch on the synthesis of desmin and light meromyosin one generation earlier than normal. Alternatively, PMA might alter the normal differentiation program of the postmitotic, mononucleated myoblast, thus allowing this type of myogenic cell to incorporate [³H]dThd one generation later than normal (22, 26, 27, 29). The observation that a small percentage of mononucleated desmin-positive cells incorporate [³H]dThd in control cultures and that approximately 5% of metaphase-arrested cells in control cultures express desmin is consistent with either interpretation and suggests that PMA may be enhancing the frequency of an event which normally occurs in myogenic cultures at a very low rate. However, it must be stressed that there is no evidence that, even in control cultures, these cells survive or replicate successfully. During myogenesis, both in vivo and in vitro, there is always a sizable number (circa 1 to 2%) of dying cells; such cells might have initiated incompatible differentiation programs (25). Finally, it is almost certain that our cultures contain a small contaminant population of smooth muscle cells included in the initial inoculum. These cells would replicate and also be desmin positive although not light meromyosin positive (2).

Attempts to elucidate the fate of the replicating desmin- and light meromyosin-positive cells in PMA cultures have also been inconclusive. When removed from the influence of PMA, many cells behave as normal myogenic cells. They withdraw irreversibly from the cell cycle, fuse, and assemble myofibrils consisting of muscle-specific, interdigitating, thick myosin and thin α-actin filaments. The occasional observations of desmin- and light meromyosin-positive cells suggest that some PMA-treated cells undergo, or attempt to undergo, karyokinesis and cytokinesis. The failure to maintain a replicating desmin- or light meromyosin-positive population through serial passage, however, argues conclusively against the possibility that the drug induces a continuously replicating population with such atypical properties. Furthermore, the modest 50 to 60% increase in desmin-positive cells with each cell cycle indicates either that few of the desmin-positive cells that incorporate [³H]dThd actually divide or that their daughter cells cease desmin expression or die. Clearly, PMA is not a mitogen for myogenic cells in the sense that it does not induce a measurable increase in the total number of definitive myoblasts. Further studies on normal and PMA-treated cultures are required to determine the developmental significance and fate of cells that are desmin and light meromyosin positive but incorporate [³H]dThd.

An alternative explanation for our results is that the observed [³H]dThd uptake by antibody-positive cells represents some form of DNA repair or gene amplification rather than premitotic S-phase incorporation (20, 51). Indeed, the unscheduled uptake of [³H]dThd reported here by desmin- and light meromyosin-positive oligonucleated myotubes in early PMA cultures (Fig. 5) is probably not normal replicative DNA synthesis and will not be followed by normal cell division (27). It may be significant in this context that PMA induces chromosomal aberrations and polyplody in mouse epidermal cells (19) and human lymphocytes (15). PMA has also been reported to induce mitotic aneuploidy in yeast (39), increase the rate of DNA breakage in human leukocytes although not in other cell types (4, 18), and increase sister chromatid exchange in Chinese hamster and mouse cells (Refs. 31, 32, and 37; see also Refs. 33 and 50). The effects of the above changes on [³H]dThd uptake, however, are not yet known.

In conclusion, we have shown that, in the presence of PMA, myogenic cultures produce mononucleated cells synthesizing both desmin and light meromyosin. These desmin- and light meromyosin-positive cells differ from normal, untreated, presumptive, or definitive myoblasts in at least 3 respects: (a) they fail to assemble normal myofibrils; (b) they do not fuse to form normal myotubes; and (c) they incorporate [³H]dThd and some enter mitosis.

The apparent dissociation of the synthesis of muscle-specific proteins and withdrawal from the cell cycle may be an infrequently exercised option in the differentiation program of normal myogenic cells, as the small number of desmin-positive cells incorporating [³H]dThd in control cultures would suggest. In this sense, PMA may be amplifying an infrequently occurring event, i.e., continued DNA synthesis following expression of muscle-specific proteins. It should be stressed that this is different from a mitotic effect described previously for PMA (36, 38, 47) in which cells that were in a stationary growth phase, but neither postmitotic nor destined to become postmitotic, were induced to initiate DNA synthesis. The continued DNA synthesis in cells that would normally become postmitotic may be relevant to the tumor-promoting ability of PMA. Epidermal cells differentiate along a pathway terminating in postmitotic, anucleate keratinocytes (42). If PMA enables some small subset of cells in this lineage to undergo extra cell divisions, the possibility of establishing a clone carrying an initiator-induced mutation would be enhanced greatly. In this regard, it is interesting that the nonpromoting phorbol ester 4-α-PDD did not induce continued DNA synthesis in cells expressing muscle-specific proteins.

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Fig. 1. Primary myogenic cultures grown in control (a to d) and PMA (e to h) medium for 3 (a, c, e, g) and 6 (b, d, f, h) days. a and c, identical microscopic fields from a control Day 3 culture stained with rhodamine-labeled anti-desmin and bisbenzimide, respectively; b and d, the same microscopic field of a Day 6 culture double-stained similarly; e and g, the same microscopic field of a PMA-treated Day 3 culture stained with rhodamine-labeled anti-desmin and bisbenzimide; whereas f and h are double-stained PMA-treated Day 6 cultures. × 195.
Fig. 2. Four-day-old primary BrdUrd cultures subcultured into either BrdUrd (a to d), control (e to h), or PMA (i to l) medium and stained with antibody to desmin and bisbenzimide on Days 2 (a, b, e, f, i, j) and 6 (c, d, g, h, k, l) after subculture. While there is no increase in the number of desmin-positive cells in the BrdUrd culture, there is a dramatic increase in their number in the control and PMA cultures, demonstrating that presumptive myoblasts will differentiate into desmin-positive cells in PMA medium. Note that several fields were scanned to locate the desmin-positive cells in a and c. Data from a separate experiment indicate that fewer than 1% of the nuclei in such cultures are in desmin-positive cells 2 days after plating. × 130.
Fig. 3. Primary PMA cultures pulsed for 45 min with $[^3H]d$Td (0.5 $\mu$Ci/ml) 36 hr after plating and processed for autoradiography and immunofluorescent localization of either desmin (a to c) or light meromyosin (d to f). a to c, Identical microscopic fields showing that 2 of the desmin-positive cells (a, arrows) have incorporated $[^3H]d$Td (b, arrows), whereas many of the other nuclei in the field have not incorporated label as shown with bisbenzimide (c). d to f, Identical microscopic fields showing a light meromyosin-positive cell (d, arrow) that has also incorporated $[^3H]d$Td (e, arrow). Bisbenzimide stain in f shows that many nuclei remain unlabeled after a 45-min pulse. × 400.
Fig. 4. PMA-treated cells double-stained with bisbenzimide and either desmin (a and b; Day 6 primary culture) or light meromyosin (c and d; Day 4 primary culture) antibodies. In the PMA-treated cultures, occasional cells in various stages of mitosis (b and d, arrows) contained desmin and light meromyosin (arrows, a and c, respectively). The fate of these cells is not known, and it is possible that they do not yield viable desmin- or light meromyosin-positive progeny. × 420.

Fig. 5. Oligonucleated muscle cell in 36-hr primary PMA culture pulsed for 45 min with [³H]dThd (0.5 μCi/ml) immediately prior to staining. a to c, identical microscopic fields from a culture stained with both anti-light meromyosin (a) and bisbenzimide (c) and viewed using bright-field illumination (b). Note the filamentous, although not striated, distribution of light meromyosin (a) and the similar labeling density of all 5 nuclei within the cell (b). (× 340).
Effects of Phorbol 12-Myristate 13-Acetate on the Differentiation Program of Embryonic Chick Skeletal Myoblasts

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