Selective effects of phorbol 12-myristate 13-acetate on myofibrils and 10-nm filaments

(myogenesis/phorbol diester/tumorogenesis/cell differentiation/immunofluorescence)

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ABSTRACT Phorbol 12-myristate 13-acetate (PMA) has a prompt and selective catabolic effect on striated myofibrils in postmitotic myotubes. Fluorescein-labeled antibodies against light meromyosin were used to follow the effects of PMA on the muscle-specific myosin in myofibrils. The response of actin filaments was monitored by decoration with heavy meromyosin. The response of the two types of 10-nm filaments in myotubes was followed by fluorescein- and rhodamine-labeled antibodies to the fibroblastic and muscle-specific filament proteins, respectively. Within 2-3 days, PMA induced dismantling of virtually every striated myofibril in every myotube in the culture. These myotubes bound little or no anti-light meromyosin, and tests to detect the α-actin filaments of the myofibrils with heavy meromyosin were negative. In contrast, the nonmuscle actin in the subsarcolemmal microfilaments persisted in PMA-treated myotubes and was decorated with heavy meromyosin. The sarcoplasmic reticulum, mitochondria, and Golgi bodies appeared normal. Myotubes depleted of myofibrils by PMA displayed large numbers of muscle-specific 10-nm filaments. This preferential degradation of the myosin and actin of the myofibrils was reversible. These myotubes formed a normal complement of myofibrils 24-48 hr after removal of PMA. When, after 3 days in PMA, the cultures were treated for an additional 3-8 days, a transitory subpopulation of PMA-resistant myotubes appeared.

Though cocarcinogens appear to be as important as carcinogens in determining where and when tumors emerge, little is known of the mechanisms of their action (1-3). Recently, phorbol 12-myristate 13-acetate (PMA) has been reported to act on the mechanisms that regulate cell differentiation (2-5). The effect of PMA on the differentiation program of the cell probably depends on whether the cell is "normal" or "transformed."]

PMA reversibly blocks terminal differentiation of normal myogenic cells (6, 7), chondrogenic cells (8), and melanogenic cells (9). PMA delays the emergence of adipocytes in a population of 3T3 cells (10) and suppresses the differentiation of normal gut and skeletal cells in sea urchin embryos (11).

The effects of PMA on transformed cells is less predictable. It blocks the induction of hemoglobin synthesis in many, but not all, lines of Friend erythroleukemic cells (12-14). Melanin synthesis is enhanced in one line of melanoma cells by PMA, whereas in another line it is delayed (15, 16). PMA, which is not toxic to normal pigmented cells, kills the melanoma cells (9). A line of promyelocytic leukemic cells treated with PMA forms macrophage-like cells (17).

This report focuses on the rapid and preferential catabolic effects of PMA on striated myofibrils in cultured postmitotic myotubes.

MATERIALS AND METHODS

Myogenic Cultures. Primary muscle cultures were prepared from 11- to 12-day chicken embryos (18). All cultures were grown in normal medium for 5 days. On day 5, PMA (0.1 μM) (Peter Borchert, Univ. of Minnesota, Minneapolis) was added to the cultures. Not all batches of PMA were equally potent, and our observations suggest that different batches of embryo extract and horse serum affect the response to PMA. Embryo extract has been shown to rapidly degrade PMA (unpublished data). Maximal effects with PMA were observed when cells were fed every 12 hr. The cells were treated with PMA for 1-20 days. The inactive analogue 4α-phorbol 12,13-didecanoate had no discernible effect. The cultures were prepared for fluorescence (19) or electron microscopy (20). To determine the presence of myofibrillar actin or actin associated with the subsarcolemmal microfilaments, the heavy meromyosin (HMM)-decoration technique was used (21, 22). DNA was measured by the method of Hinegardner (23).

Visualization of Antibody-Treated Cells. Fluorescein-labeled monospecific antibody to adult chicken pectoral light meromyosin (anti-LMM) was prepared by affinity chromatography (24, 25). Ouchterlony diffusion tests demonstrated that this anti-LMM formed a precipitin band against 0.6 M KI extracts of adult and cultured embryonic chicken pectoralis muscles. It formed an spur with cardiac myosin, indicating partial homology, but it did not cross react with myosin from (i) replicating presumptive myoblasts, (ii) smooth muscle, or (iii) fibroblasts, nerve cells, chondroblasts, etc. (5, 6). The preparation and specificities of the two types of antisera to the two types of 10-nm filaments—the "fibroblastic type" and the "muscle-specific type"—have been described (19, 26-28). Because the size of the 10-nm filament subunit varies not only with cell type but also with species, we now prefer a nomenclature that refers to the cell type from which the subunit was isolated, rather than the subunit size. Therefore, our antisera against the subunit of chicken fibroblast 10-nm filaments (FF), previously referred to as "anti-58K" will now be termed "anti-FF" (anti-fibroblastic filament subunit), and antibodies to the muscle-specific 10-nm filament subunit (MF) previously referred to as "anti-55K" will now be termed "anti-MF" (anti-muscle-specific subunit). Anti-FF, termed "anti-vimentin" by Franke et al. (29, 30), was used in the indirect immunofluorescence technique (0.1 mg/ml) and visualized with a 1:20 dilution of fluorescein-labeled goat anti-rabbit IgG (Antibodies, Inc.). The anti-MF, termed "anti-desmin" by Izant and Lazarides (31) and "anti-skeletin" by Small and Sobieszek (32), was directly conjugated with rhodamine and used as a concentration of 0.5 mg/ml. To reduce nonspecific binding of labeled antibodies, some preparations were washed with a 0.5%
Triton X-100/0.1% sodium dodecyl sulfate saline solution, pH 7.4, or 0.01 M Tris-buffered saline, pH 9.5 (19).

RESULTS

Selective Disassembly of Striated Myofibrils by PMA. Day 5–8 myogenic cultures contain thousands of postmitotic multinucleated myotubes in addition to many replicating presumptive myoblasts and fibroblasts. The myotubes contract spontaneously. They range in length from 0.1 mm to over 2.0 mm and in width from 5 to over 40 μm, and they contain from 2 to over 100 nuclei per myotube. Every myotube has large numbers of striated myofibrils; larger myotubes have more myofibrils than smaller ones (Fig. 1 a and b). The proteins composing the myofibrils can be distinguished by their respective molecular weights and immunological properties from the contractile proteins (i) in their mother cells, the presumptive

![Micrographs](image-url)

**FIG. 1.** (a–c) Micrographs of the identical microscopic field of a normal day 6 culture. (a) A phase-contrast micrograph revealing how the density of mononucleated cells above, below, and between the striated myotubes tends to obscure their myofibrils. (b) Same field, showing the localization of the fluorescein-labeled anti-LMM. Every single myotube in the culture displays numerous aligned striated myofibrils. The replicating presumptive myoblasts and fibroblasts do not bind the muscle-specific anti-LMM. (c) Same field, showing the localization of the muscle-specific rhodamine-labeled anti-MF. These myotubes were selected to demonstrate that as early as day 6 the shift of the MF protein from its longitudinal orientation to its final localization in the I-Z region has begun. See ref. 27 for further details on the behavior of the 10-nm filament antigens. (d–f) Micrographs of the identical microscopic field of a day 6 culture exposed to PMA for the previous 24 hr. (d) A single striated myotube can barely be detected among the extraordinarily elongated, mononucleated, presumptive myoblasts and fibroblasts. However, as shown in e and f, other myotubes are in fact present in this field. (e) Same field, showing the localization of fluorescein-labeled anti-LMM. Note the modest loss in alignment of striations in the large horizontal myotube and the total absence of fluorescence in the smaller myotube, indicated by the arrow. (f) Same field, showing the localization of rhodamine-labeled anti-MF. Note its longitudinal localization in the myotube that has still retained some myofibrils, as well as in the myotube totally depleted of them (arrow). (×115.)
myoblasts, and (ii) in fibroblasts (Fig. 1 b and c) or other nonmyogenic cells (5, 6, 21, 33–35).

PMA has a prompt effect on these myotubes. Day 6 myotubes exposed to PMA for the previous 24 hr cease contracting. Under the phase-contrast microscope they appear flatter, shorter, and broader, and they display scalloped borders. The preferential disruption of the myofibrils, as revealed by staining with anti-LMM, is apparent by 24 hr (compare Fig. 1 a and b with d and e), and after 72 hr in PMA almost all myotubes (≈90%) had been depleted of most of their myofibrils (Fig. 2a). Many treated myotubes approach the morphology of the "myosacs" induced by Colcemid (18). The dull to negative staining with anti-LMM suggests a great diminution in the amount of myosin in myotubes treated with PMA for 72 hr, confirming our electron microscopic studies (Fig. 2c and ref. 20). Efforts to detect HMM-decoratable \( \alpha \)-actin filaments—possible remnants of the dismembered myofibrils—were negative. In contrast, arrowhead complexes after decoration with HMM formed among the microfilaments subtending the sarcolemma. Clearly, the different types of actin present in myotubes respond differently to PMA.

This short-term effect of PMA is entirely reversible. Striated myofibrils binding the anti-LMM, which can also be observed under the phase-contrast microscope, are apparent within 24–48 hr after removal of PMA from cultures that had been in the cocarcinogen for 72 hr (Fig. 3). These myotubes with de novo assembled myofibrils contract spontaneously and most have lost their "myosac" configuration.

**Effects of PMA on 10-nm Filament and Other Organelles.**

Modest numbers of 10-nm filaments course parallel to, and between, the striated myofibrils in control day 5–8 myotubes (26–28, 36). Two classes of 10-nm filaments are present: "fibroblastic" filaments found in many types of cells (4, 19, 26–29) and "muscle-specific" filaments (19, 26–28, 30–32, 37). Binding of the anti-FF to the longitudinally oriented 10-nm filaments in control day 4–5 myotubes is prominent, whereas the antigen is difficult to detect in control day 8 myotubes. In contrast, the anti-MF still displays a longitudinal orientation in control day 6 myotubes, but in approximately 30% it is beginning to localize in the I-Z region as well (Fig. 1c). The spatial redistribution continues, and in control day 8 myotubes the MF antigen is prominently associated with most I-Z bands. Modest numbers of postmitotic mononucleated myoblasts bind the anti-MF, demonstrating that fusion is not a precondition for initiating the synthesis of muscle-specific proteins (4, 5, 28, 33, 35, 38).

PMA has no obvious effect on the distribution of the FF protein. It disappears in the PMA-treated day 8 myotubes as it does in normal day 8 myotubes. After 1–3 days in PMA, the anti-MF binds to longitudinally oriented filaments, stains irregular patches, and is distributed throughout every myotube and "myosac" that has been depleted of its myofibrils. This

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**FIG. 2.** Micrographs of myotubes that had been exposed to PMA for the previous 3 days. (a) The dull fluorescence after staining with anti-LMM is slightly above background, consistent with the finding that most thick filaments have disappeared in these myotubes (see figure 2c and figure 3 in ref. 20). (b) Same myotube as in a, stained with rhodamine-labeled anti-MF. Note the irregular borders of the PMA-treated myotube as well as the intense staining of the fine pseudopodial processes. The mononucleated cells present in this microscopic field that do not bind either antibody form a dense mat of cells similar to that in Fig. 1d. (×115.) (c) Electron micrograph of a myotube that had been in PMA for the previous 3 days. Strewn among the 10-nm filaments are normal-appearing mitochondria, sarcoplasmic reticulum, and Golgi bodies as well as a small cluster of randomly oriented interdigitating thick and thin filaments (arrow). (×21,000.)

**FIG. 3.** Fluorescence micrograph of a culture that had been in PMA for 3 days and then was transferred to normal medium for 48 hr and stained with anti-LMM. The rapidity of reassembly of the striated myofibrils is striking. (×115.)
staining extends into the finest pseudopodia (Figs. 1f and 2b). The density of the 10-nm filaments in these treated myotubes is extraordinary (Fig. 2c).

That the effects of PMA are not generally cytotoxic is indicated by the following: (i) In three different experiments the ratio of PMA-treated to control cultures varied between 1.1 and 1.2 in terms of total DNA; and (ii) the number of myotubes in 20 randomly counted microscopic fields yielded ratios of PMA-treated to control myotubes of 0.9; over 300 myotubes were counted in each of these experiments.

Generation of PMA-Resistant Myotubes. When day 8 myotubes that have been in PMA for 3 days are cultured in PMA for an additional 3–8 days, a sizable subpopulation of PMA-resistant myotubes emerges. These myotubes have striated myofibrils (Fig. 4a), but often their Z-bands are wanting in electron-dense material (data to be published elsewhere). The distribution of the anti-FF in these resistant myotubes is obscured, owing to the overlay of fluorescent mononucleated cells (e.g., Fig. 1d). However, the anti-MF is bound largely at the growth tips of these myotubes (39). Longer exposure to PMA results in a precipitous loss of both the “resistant” myotubes and the flattened myosac-like structures. The disappearance of the “resistant” myotubes, their atypical Z-bands, and the atypical distribution of the anti-MF indicate that the PMA-resistant myotubes differ from myotubes never exposed to PMA. Cultures in PMA for 6–11 days also display many (i) multinucleated “myosacs” that do not bind any anti-LMM but bind intensely the anti-MF (Fig. 4) and (ii) postmitotic mononucleated myoblasts that bind the anti-MF, which may be the cells that fuse to form the generation of PMA-resistant myotubes. Cultures in PMA for 6–11 days can form multinucleated myotubes when subcultured and reared in normal medium. Clearly, protracted exposure to PMA does not affect the multiplication of presumptive myoblasts.

**DISCUSSION**

The notion that agents that transform normal cells into neoplastic cells tend, to various degrees, to interfere with the mechanisms regulating normal cell differentiation is not novel (40–43). That myogenic, chondrogenic, and melanogenic cells infected with a temperature-sensitive mutant of Rous sarcoma virus fail to differentiate at permissive temperature, but differentiate at nonpermissive temperature, is consistent with this view (44–46). This report, plus the observations that PMA blocks the differentiation of normal cartilage (8) and normal pigment cells (4, 5, 9), also supports this view.

PMA does not block metabolic pathways indiscriminately. Chondroblasts in PMA are selectively but reversibly inhibited from synthesizing their unique phenotypic molecules—type IV sulfated proteoglycans and type II collagen chains—whereas their replication is enhanced (8). Similarly, PMA selectively, but reversibly, inhibits the synthesis of melanosomes and melanin in normal pigment cells without altering their replication (9). PMA, then, does not interfere with the myriad of household molecules required for growth and replication. However, like pp60src protein kinase at permissive temperature (4, 44–46) or BrdUrd incorporation into DNA (5, 47, 48), PMA does not render myogenic, chondrogenic, or melanogenic cells more “embryonic” or change their status in their respective lineages. Though blocking expression of the differentiation program of each of these cell types, these molecules do not interfere with the genetic mechanisms ensuring that the appropriate differentiation programs are transmitted to descendant cells with great fidelity (4, 5, 8, 9).

Failure of myotubes in PMA to stain with anti-LMM could be due to (i) the dispersal of thick filaments to myosin monomers at concentrations too low to be visualized (37), or (ii) their degradation to peptides or even to amino acids. Failure to induce cytoplasmic arrowhead complexes with HMM similarly suggests that the α-actin filaments were degraded either to G-actin or to amino acids. The mechanism(s) by which PMA ultimately induces the disassembly of myofibrils is unknown. However, preliminary experiments have shown that the reemergence of myofibrils in cultures treated with PMA for 3 days does not occur in the presence of cycloheximide during recovery. These findings are also consistent with the notion that the proteins of the original myofibrils were degraded into amino acids in PMA and the new population of myofibrils depends on de novo protein synthesis. Data on the synthesis of the muscle-specific myosin light chains and α-actin indicate a diminution in their synthesis in the presence of cycloheximide (unpublished data). If the rate of synthesis of the MF protein is altered in PMA or its degradation is greatly reduced. Lastly, the observation that the α-actin could not be decorated with HMM, in contrast to the α-actins in the subarcosomal microfilaments, underscores the differences in behavior of the different actins in the same myotube (see also ref. 25).

Of interest is the generation of PMA-resistant myogenic cells. Many types of cells inactivate PMA (49). Protracted exposure may reduce the number of available receptors (50) or even induce the myogenic cells to accelerate their inactivation of the
cancergen. Whether the PMA-resistant myotubes represent a new generation of postmitotic myoblasts that fused late in the culture period or the recovery of myotubes that had been depleted of their myofibrils remains to be clarified. In either case, two distinctly different types of inhibitory effects of PMA on myogenesis—namely, blocking fusion of myoblasts into myotubes (6) and blocking the formation and maintenance of myofibrils—can be reduced after protracted exposure to the cocarcinogen.

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