Differentiated membrane specializations and myofibrillar breakdown and recovery in cultured adult cardiac myocytes treated with TPA and diacylglycerol

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Summary

Cultured adult rat ventricular cardiac muscle cells were treated with either 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or diacylglycerol (DAG) and observed by in situ transmission electron microscopy. Membrane specializations present in untreated cells (intercalated discs, transverse tubules, plasmalemmal couplings) were also present after TPA and DAG treatment. In the case of the transverse tubular system, there was morphological evidence for active growth. Our studies showed that myofilaments began to become disorganized after 12–24 h of TPA treatment and that after 2 days of exposure to TPA the breakdown of sarcomeres was essentially complete. Myocytes that were treated with TPA for 2 days and then allowed to recover in control medium for 5 days contained sarcomeres in various stages of reassembly. These data indicate that TPA-treated cardiac myocytes retain several membrane specializations, suggesting that there are separate controls for myofilament organization and the maintenance of these differentiated plasmalemmal regions. Furthermore, the ability of the myocytes to recover from TPA treatment may provide investigators with a useful model with which to study myofibrillogenesis.

Key words: cardiac myocyte, ultrastructure, tissue culture, phorbol ester.

Introduction

In recent years cultured cardiac myocytes derived from a variety of adult mammalian species (Jacobson, 1977; Claycomb & Palazzo, 1980; Nag & Cheng, 1981; Moses & Claycomb, 1982a, 1984; Claycomb & Moses, 1985; Cooper et al. 1986) have proved to be useful experimental systems for the in vitro study of cardiac muscle. Cardiac myocytes maintained in long-term culture provide a system in which the physiology and morphology of the cells potentially can be manipulated. Such experiments may yield insight into the mechanisms responsible for the normal and pathological development and function of cardiac muscle cells.

We have recently shown that the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA), as well as diacylglycerol (DAG), stimulates DNA synthesis and protein accumulation, and alters the morphology of cultured adult rat ventricular myocytes (Claycomb, 1988a; Claycomb & Moses, 1988; Moses & Claycomb, 1988). Morphological alterations in treated myocytes included cellular enlargement and, at the ultrastructural level, myofibrillar disorganization. These findings are similar to those of Holtzer and his colleagues (Cohen et al. 1977; Croop et al. 1982; West & Holtzer, 1982; Dlugosz et al. 1983; Holtzer et al. 1985; Antin et al. 1986; Lin et al. 1987) on the effects of TPA on cultured skeletal muscle. TPA presumably causes these effects on cytoskeletal structures by binding to and activating protein kinase C, which, by virtue of its ability to phosphorylate the serine and threonine residues of numerous proteins, can influence a number of metabolic pathways by altering gene expression (see Nishizuka, 1984, for review). The effect of TPA on gene expression appears to be dependent on the stage of differentiation of cultured skeletal muscle cells (Holtzer et al. 1973; Cohen et al. 1977; Croop et al. 1982; Antin et al. 1986; Cassa et al. 1988).

The breakdown of sarcomeric organization in phorbol ester-treated cardiac myocytes provided an opportunity of investigating cardiac muscle cell membrane specializations such as couplings, transverse (T) tubules, and the intercalated disc (ID) in the absence of well-organized myofibrils. We were also interested in obtaining a rough estimate of the time course of TPA-induced myofibrilar...
breakdown and in assessing the ability of the myocytes to reorganize sarcomeres after removal of TPA. Such an ability might support the use of cardiac myocytes recovering from phorbol ester treatment as a model for cardiac muscle cell differentiation.

Materials and methods

Culture techniques

Ventricular cardiac muscle cells were isolated from adult (200–250 g) female Holtzman rats and cultured as described (Claycomb & Palazzo, 1980; Claycomb & Lanson, 1984). Briefly, myocytes were isolated by retrograde aortic perfusion with collagenase in Joklik’s medium. Cells of four hearts were pooled, and approximately 3–5 × 10⁶ cells were plated in 25 cm² plastic tissue culture flasks (precoated with rat tail collagen) containing 5 ml of rabbit corneal cell-conditioned medium with 10% fetal bovine serum, 2x vitamins, non-essential amino acids, penicillin (100 units ml⁻¹), streptomycin (100 µg ml⁻¹), human transferrin (10 µg ml⁻¹), bovine insulin (10 µg ml⁻¹), and trace minerals (Claycomb & Lanson, 1984). Non-muscle cells were eliminated from the cultures by adding cytotoxic-1-β-D-arabinofuranoside (14 µg/ml) to each flask on the first and third days of culture. The medium was not changed during the first 7 days of culture. On the seventh day, and every other day thereafter, the medium was changed, and the cells were cultured in MEM with 2x vitamins, non-essential amino acids, trace minerals, 10% fetal bovine serum, penicillin and streptomycin. Cells were treated with TPA or DAG 7–10 days after their initial isolation.

Cells were treated with medium containing TPA (50 ng ml⁻¹) in 0.02% dimethylsulfoxide (DMSO) or DAG (50 µg ml⁻¹) in 0.01% ethanol. The control medium for the TPA treatment was 0.02% DMSO in normal medium, and the control for DAG treatment was 0.01% ethanol in normal medium. In addition, some cultures were treated with the non-tumour-promoting phorbol ester α or β analogs (100 µg ml⁻¹) in 0.02% DMSO.

Electron microscopy

Cultured myocytes were processed for transmission electron microscopy (TEM) as described (Moses & Claycomb, 1982a; Moses et al., 1988, 1989). Briefly, the cells were fixed with 4% glutaraldehyde in 0.1 M-sodium cacodylate buffer, postfixed in either 1% osmium tetroxide or 1% osmium tetroxide plus 0.8% potassium ferrocyanide in the same buffer, en bloc stained in 0.5% aqueous uranyl acetate, dehydrated in an ascending series of ethanol, infiltrated in Epon–Araldite epoxy resin, and heat polymerized. Cells were prepared for both en face and transverse (with respect to the culture substratum) thin-sectioning (Moses et al., 1988, 1989). Pale gold to silver sections were cut on a Reichert OMU3 ultramicrotome, retrieved on uncoated 200 mesh copper grids, post-stained with lead citrate, and examined with a Philips 300 TEM at an operating voltage of 60 kV. Images were recorded on Kodak Electron Microscope Film.

Results

General ultrastructure of TPA-treated myocytes

As previously reported (Claycomb & Moses, 1988; Moses & Claycomb, 1988), TPA dramatically decreased myofibrillar organization. Fig. 1 illustrates examples of control and TPA-treated myocytes. DAG-treated myocytes responded similarly to those treated with TPA, except that myofibrillar disruption was not as complete at the DAG concentrations used in this study. All control cultures including DMSO, the α and β inactive phorbol ester analogs (controls for the TPA experiments), as well as cells cultured with ethanol, appeared similar to the cell illustrated in Fig. 1. These control myocytes were characterized by well-organized myofibrils, as well as other ultrastructural indicators of well-differentiated ventricular myocytes (Moses & Claycomb, 1982a).

Although all cells treated with TPA for 7 days lacked sarcomeric organization, the response of the cytoskeleton to the chemical was still ultrastructurally variable. Most cells treated with TPA responded in a manner similar to the myocyte illustrated in Fig. 1B, the cytoplasm having only occasional remnants of myofibrils. Instead, the most prominent cytoplasmic components were irregularly arranged mitochondria, sarcotubular elements, and an amorphous ground substance. Other myocytes contained large numbers of intermediate filaments or numerous microtubules. The microtubules were often in parallel arrangement. Leptomeres (see Fig. 4B) were also more common in treated cells than in controls. Maximal sarcomeric organization in TPA-treated myocytes consisted of isolated linear arrays of loosely organized myofilaments associated with irregular densities corresponding to Z lines. Although both thick and thin filaments were present, sarcomeric banding patterns were absent (Claycomb & Moses, 1988; Moses & Claycomb, 1988).

Membrane specializations in TPA- and DAG-treated myocytes

The lack of organized myofibrils in the TPA- and DAG-treated muscle cells provided an opportunity of studying cardiac muscle plasmalemmal specializations in the absence of sarcomeric organization. Regions of cells containing areas resembling intercalated discs (IDs) were easily located. These areas were similar to previously reported IDs in cultured ventricular muscle cells (Moses & Claycomb, 1982a, 1984; Simpson & Savion, 1982; Moses & Delcarpio, 1983) except that the junctional complexes were anatomically simpler (Figs 1 and 2). The intermediate junction (fascia adherentes) portion of the ID had a subplasmalemmal density of variable prominence and was associated with an array of intermediate filaments arranged roughly parallel to the plasmalemma (Figs 2 and 4). Since sarcomeres were effectively absent in TPA-treated cells, IDs did not serve consistently as terminal Z lines.

Myocytes maintained an intercellular space of at least 20 nm except for areas of close contact that were identified as gap junctions. Gap junctions were present between TPA- and DAG-treated myocytes in which myofibrils were completely disorganized (Fig. 2) and could easily be located in both en face and transverse sections.

Considering the level of myofibrillar disorganization, T tubes were surprisingly common in treated myocytes (Fig. 3). When present, T tubes were often, although not
exclusively, present near irregular Z densities. Although no attempt was made to compare T tube volumes quantitatively, they appeared to be more common in DAG- than TPA-treated myocytes.

Formation of T tubules (tubulogenesis) appeared to be active in both TPA- and DAG-treated cells. Numerous caveolae were found fused with T tube membranes, suggesting that the tubules were enlarging through the process of repeat caveolation (Ishikawa, 1968; Moses & Kasten, 1979; Moses & Claycomb, 1982b, 1985). Elements of the Golgi apparatus and associated lateral vesicles were often in close proximity to these T tubes (Fig. 3).

Both TPA- and DAG-treated myocytes contained both peripheral (plasmalemma-associated) and internal (T tube-associated) junctional sarcoplasmic reticulum (PJSR and IJSR, respectively) (Figs 2 and 3) (Sommer & Johnson, 1979).

**Fig. 1.** A. Control myocyte, 17 days in culture. Note the well-organized myofibrils (arrows). Most of the cytoplasm is occupied by sarcomeres (osmium ferrocyanide postfixation). B. TPA-treated (10 days in normal culture, 7 days TPA treatment) myocyte. Note the absence of myofibrillar structure. The intercalated disc (arrow) is illustrated at higher magnification in Fig. 2. Osmium ferrocyanide postfixation. $\times 7000$.
Fig. 2. Intercalated discs and intercellular junctions in TPA-treated myocytes. A relatively complex disc is illustrated in A. Note that the disc is not an area of consistent myofilament insertion. Gap junctions are indicated by arrows. B. Intercalated disc containing both intermediate (open arrow) and gap (filled arrow) junctional components. A coupling (arrowhead) is also present. The gap junction is illustrated at higher magnification in the inset. C. Gap junction (arrow) between two transversely sectioned myocytes; arrowhead, growth surface. All micrographs, osmium ferrocyanide post fixation. A, ×70 900; B, ×76 000; inset ×171 000; C, ×110 700.
Fig. 3. T tubes (*) in TPA- (A,B) and DAG-treated (C) myocytes. Note that couplings (open arrows) are present. Stacks of Golgi cisternae (arrow in C) are frequently found near tubules with fused caveolae. Note the amorphous material present in the T tube lumen (arrowheads). All cells sectioned \textit{en face} except B, which was sectioned transversely. A, C. Osmium ferrocyanide postfixation; B, osmium tetroxide postfixation. A, \times 58,500; B, \times 2900; C, \times 57,000.

\textit{Time course of TPA-induced myofibrillar changes}

TPA-treated myocytes were fixed after 4, 12, 24, 36 and 48 h in culture in order to determine the time course of the effect of TPA on myofibrillar organization. These time-course studies demonstrated that TPA did not induce myofibrillar changes in a synchronous manner. Instead, different cells from the same culture flask displayed varying levels of sarcomeric organization. In fact, within the same cell some regions exhibited high levels of sarcomeric organization while other regions contained only irregularly arranged myofilaments.

Cells fixed 4–12 h subsequent to TPA treatment (not illustrated) were essentially similar to control cells, although some regions of some cells appeared to be beginning to lose sarcomeric integrity. Most myocytes fixed 24–48 h after treatment, however, were obviously morphologically altered. These ultrastructural modifications resulted in cells with morphological characteristics intermediate between control cells and those treated with TPA for 7 days.

The earliest sign of impending myofibrillar disorganization was the appearance of irregular Z lines and a coordinate loss of regular sarcomeric structure (Figs 4 and 5). Associated with this altered myofibrillar structure...
was the appearance of numerous free cytoplasmic ribosomes. IDs remained intact while sarcomeric structure was being altered (Figs 2 and 4).

As myofibrillar disorganization continued, sarcomeric structure became increasingly irregular. In the earlier stages, sarcomeres were still recognizable by virtue of their irregular Z lines and myosin filaments. More completely disorganized cells also exhibited irregularities in actin, intermediate and myosin filament arrangements. Intermediate filaments were encountered more frequently as the cells underwent myofibrillar disorganization and were found both within and at the periphery of sarcomeres as well as in free cables. Myosin thick filaments became scarcer and more irregularly located as disorganization proceeded (Fig. 5). However, even in profoundly disorganized cells, myosin molecules were still found associated with irregular Z lines and the terminal densities of intercalated discs (Figs 4 and 5).

After 2 days of TPA treatment, most of the myocytes were devoid of sarcomeric organization (Fig. 6) and resembled cells cultured with TPA for 7 days. Myocytes rarely contained a few organized sarcomeres. However, even the most extensively organized myocytes after 2 days of TPA treatment had far fewer and less-well-organized sarcomeres than did control myocytes. After 4 days of TPA treatment organized sarcomeres were exceedingly rare, and myocytes appeared similar to those cultured in TPA for 7 days (Claycomb & Moses, 1988; Moses & Claycomb, 1988).

Recovery from TPA treatment

In order to determine whether or not cultured adult

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Fig. 4. A,B. Myocytes treated with TPA for 24 h. Note that the cell in A exhibits considerably greater sarcomeric organization than the cell illustrated in B. Filled arrows, sarcomeres; open arrow, intercalated disc; *, leptomeric complexes. Both micrographs, osmium ferrocyanide postfixation. A, ×7950; B, ×9700.
ventricular myocytes were able to recover from the effects of TPA treatment, myocytes were cultured for 2 days in the presence of TPA, followed by culture in TPA-free medium for 5 days. Myocytes treated in this manner demonstrated ultrastructural evidence of sarcomerogenesis (Fig. 7), and had considerably more and better organized sarcomeres than did myocytes fixed and examined after 2 days of TPA treatment. Even though myofilament organization and, presumably, synthesis (as evidenced by the presence of numerous ribosomes associated with the filaments) were active, myocytes did not regain sarcomeric organization equivalent to that observed in control myocytes cultured for the same length of time in the absence of TPA.

**Discussion**

In many respects cultured adult rat cardiac myocytes responded to TPA and DAG treatment similarly to cultured embryonic chick skeletal myotubes as described by Holtzer and his co-workers (Cohen et al. 1977; Croop et al. 1982; West & Holtzer, 1982; Dlugosz et al. 1983; Holtzer et al. 1985; Antin et al. 1986; Lin et al. 1987). Their studies have shown that in cultured skeletal muscle cells TPA induced a breakdown of organized sarcomeres and an accumulation of intermediate filaments. At the biochemical level, the synthesis of a number of muscle-specific proteins including γ-actin, muscle-specific myosin heavy and light chains, troponyosin and troponin C was inhibited. The synthesis of other cytoskeletal proteins such as α- and β-actin, non-myofibrillar myosin heavy and light chains, tubulin, vimentin and desmin, however, was not inhibited (Holtzer et al. 1985; Lin et al. 1987). These workers have proposed that TPA uncouples the coordinate synthesis of various cytoskeletal proteins, resulting in altered cytoskeletal relationships. The presence of large groups of intermediate filaments and numerous microtubules in the TPA-treated cultured adult cardiac myocytes (Claycomb & Moses, 1988; Moses & Claycomb, 1988) supports this conclusion.

The differentiation-dependent response to TPA (Holtzer et al. 1973; Cohen et al. 1977; Croop et al. 1982; Antin et al. 1986; Cassa et al. 1988) may explain why our results on cultured adult mammalian myocytes (Clay-
Our ultrastructural studies indicate that the initial site of sarcomeric disruption is the Z line (Fig. 5). This differs from results obtained in cultured TPA-treated embryonic chick skeletal myotubes in which the initial result of TPA treatment is the redistribution of actin filaments into actin 'bagels' (Lin et al. 1987). However, some mechanistic similarities for sarcomeric breakdown in myocytes and myotubes are indicated, since both cell types accumulate intermediate filaments and go through a transient stage in which myofilaments are loosely associated with irregular, punctate accumulations of Z material (Fig. 5) (Toyama et al. 1979; Lin et al. 1987; Claycomb & Moses, 1988; Moses & Claycomb, 1988).
These results support the proposal of Holtzer and his coworkers (Holtzer et al. 1985; Lin et al. 1987) that TPA induces sarcomeric breakdown by uncoupling the coordinate synthesis of myofibrillar proteins. In the case of cardiac myocytes, this may mean that the synthesis of α-actinin or other Z line proteins is increased. Alternately, the primary effect may be on actin distribution, and the altered morphology of Z lines in TPA-treated myocytes may be a secondary effect. The increased number of leptomeric complexes (Fig. 4) (Claycomb & Moses, 1988) may also be a reflection of altered actin–Z line relationships. The hypothesis that altered synthetic capabilities are responsible for sarcomeric breakdown is also supported by the altered RNA and protein contents of the treated cells (Claycomb, 1988a; Claycomb & Moses, 1988).

It has also been suggested (Lin et al. 1987) that TPA may affect sarcomeric organization by activation via phosphorylation of a neutral protease associated with the Z line (Reville et al. 1970; Busch et al. 1972; Reddy et al.)
cultured adult cardiac muscle cells are at least partially still present. SARCOMERIC ORGANIZATION IN THE RECOVERING CELLS NEVER SUGGESTS THAT THESE STRUCTURES ARE NOT DEPENDENT ON ENZYMES-INDUCED ‘ROUNDING UP’ (MOSES & CLAYCOMB, 1982b). In contrast to the altered morphology of various cytoskeletal structures, TPA- and DAG-treated myocytes retained a number of cardiac myocyte membrane specializations, including IDs, T tubes, and both internal and peripheral couplings (Figs 2 and 3). The maintenance of these structures in the absence of myofibrillar organization suggests that these structures are not dependent on myocardial cytoskeletal organization for their continued existence. Whether or not membrane specializations such as couplings and elements of the T system function normally in phorbol ester-treated myocytes is unknown, but presents an interesting avenue for further investigation. Likewise, the developmental dynamics and molecular composition of these structures represent important directions for future studies.

Although we have not quantified the surface area occupied by gap junctions (Fig. 3) in TPA- and DAG-treated compared to control myocytes, these structures were no more difficult to locate in treated than in control cells. Therefore, we assume that they occur with approximately the same frequency in treated and untreated myocytes.

With respect to electrical junctions, cultured adult cardiac muscle cells may differ from many other cell types, since in both primary epithelial (Tachikawa et al. 1986) as well as various permanent cell lines (Enomoto et al. 1981; Yancy et al. 1982) phorbol esters cause a dramatic decrease in the surface area occupied by gap junctions. Since skeletal muscle cells do not have gap junctions and since similar ultrastructural studies on cultured smooth muscle cells are not available, it is not known whether maintenance of gap junctions is a cardiac muscle-specific phenomenon.

The T system was proliferating in the treated cells as demonstrated by the numerous fused caveolae found associated with the tubules. The proximity of stacks of Golgi cisternae to actively enlarging T tubes (Fig. 3) suggests a direct relationship between enlargement of the T system and Golgi activity. Moreover, the presence of amorphous material within the lumen of T tubes (Fig. 3) further suggests that as vesicles fuse with the T tube membrane, secretory products may also be introduced into the lumen and extracellular space. This supposition is supported by the finding that TPA and DAG stimulate preproenkephalin and atrial natriuretic factor (ANF) messenger RNA expression, as well as the secretion of enkephalin and ANF into the culture medium (Springhorn & Claycomb, 1989; Claycomb, 1988b). Such amorphous structures are also found in the T tubes of untreated cultured cardiac myocytes (Moses & Claycomb, 1982a, 1984, 1985). Collectively, these findings suggest that T tubes may serve both as a mechanism for increasing the surface area of the myocyte and for ensuring the proximity of sarcomeres to the cell surface (Sommer & Johnson, 1979), as well as serving as a conduit for secretory activity. If, as seems likely, the Golgi apparatus supplies new membrane to the T system and if T tubes are indeed involved in the secretion of myocyte biosynthetic products, then microtubules may also be expected to play an important role in the geometry of T tube placement and conveyance of both new membrane and secretory products to the T tubes (Kroenbush & Singer, 1987).

In summary, TPA- and DAG-treated cultured adult mammalian myocytes provide a system in which cellular structure and function can be drastically and reversibly altered. The retention of membrane specializations and, in the case of T tubes, their continued growth suggest that such structures are neither dependent upon myofibrillar organization for their biogenesis and maintenance nor subject to the same genetic control as the various affected cytoskeletal proteins. This system should prove valuable in further studies of the mechanisms important in cardiac myocyte differentiation.

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