

Growth Factors and TPA Stimulate DNA Synthesis and Alter the Morphology of Cultured Terminally Differentiated Adult Rat Cardiac Muscle Cells

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Previous studies have established that the terminally differentiated ventricular cardiac muscle cell of the adult rat reinitiates semiconservative DNA replication when grown in culture (W. C. Claycomb and H. D. Bradshaw, Jr., 1983, *Dev. Biol.* **90**, 331-337). Work reported here shows that several growth factors and chemicals will stimulate this DNA synthetic activity in a concentration-dependent manner. Autoradiographic experiments establish that this stimulated DNA synthesis is due to cells not previously synthesizing DNA being induced to enter the S phase of the cell cycle. By far the greatest stimulation (250%) is observed with the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Fifty ng/ml is the optimal concentration, and the maximal effect is observed 5 days after adding TPA. TPA also substantially increases the protein content of the cultured myocytes. Diacylglycerols (DAG) induce these same changes, indicating that the effect of TPA is mediated by protein kinase C. The morphology of the cultured cardiac muscle cells is profoundly altered by TPA and DAG. TPA- and DAG-treated myocytes spread more thinly on the surface of the culture flask, acquire multiple nuclei, and undergo nucleolar fragmentation. The myofibrillar ultrastructure of the treated cells becomes almost totally disorganized, and intermediate filaments and rough endoplasmic reticulum accumulate in the cytoplasm. These TPA results suggest a possible relationship between the degree of ultrastructural differentiation of the ventricular cardiac muscle cell and DNA synthetic activity. This easily altered cellular plasticity should be very useful for studies of the regulation of cardiac muscle cell proliferation and cell differentiation. © 1988 Academic Press, Inc.

INTRODUCTION

We have previously observed that terminally differentiated adult rat and monkey atrial and ventricular cardiac muscle cells reinitiate semiconservative DNA replication when grown in culture (7, 9); a capacity previously thought to have been permanently and irreversibly lost during early neonatal development. These myocytes also reacquire the activities of DNA enzymes which are needed to replicate DNA (7). Autoradiographic studies by Nag and Cheng (22) have also shown that cultured adult rat ventricular cardiac myocytes incorporate [³H]thymidine into DNA, as evidenced by the concentration of silver grains over the nuclei. Cantin *et al.* (3) have reported that cultured adult rat atrial myocytes are also capable of DNA synthesis. The studies reported here were initiated to determine if this DNA synthetic activity in cultured ventricular cardiac muscle cells could be stimulated and to explore the mechanisms involved with the reinitiation of DNA replication in these terminally differentiated myocytes.

MATERIALS AND METHODS

Cell Isolation and Culture

Ventricular cardiac muscle cells were isolated from adult (200-250 g) female Holtzman rats and cultured exactly as previously described (8, 10). Briefly, the cells

were isolated by retrograde perfusion of isolated hearts via the aorta with collagenase in Joklik's medium. Cells of four individual hearts (approximately 20 million) were pooled, and approximately 3.5×10^6 cells were placed into 25-cm² plastic culture flasks (precoated with rat tail collagen) containing 5 ml rabbit corneal cell-conditioned medium with 10% fetal bovine serum, 2× vitamins, nonessential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml), human transferrin (10 µg/ml), bovine insulin (10 µg/ml), and trace minerals (8). Fibroblasts and other nonmuscle cells were eliminated from the cultures by adding cytosine-1-β-D-arabinofuranoside (14 µg/5 ml) to each flask on Days 1 and 3 of culture (10). This medium was not changed during the first 7 days of culture. On Day 7 of culture, and every other day thereafter, the medium was changed and the cells were cultured in MEM with 2× vitamins, nonessential amino acids, trace minerals, 10% fetal bovine serum, penicillin, and streptomycin. The resultant well-differentiated ventricular cardiac muscle cells (10, 19) were usually used on Day 7 or 8 of culture.

Measurement of [³H]Thymidine Incorporation into DNA

Ventricular cardiac muscle cells were cultured with 2 µCi/ml [³H]thymidine either continuously for the specified period of time or for 24 hr. When the cells were

cultured continuously with [³H]thymidine, the medium was changed every 48 hr, and fresh label was used. Following the labeling period, the cells were trypsinized and briefly centrifuged in a microfuge, and the resulting pellet was sonicated in phosphate-buffered saline. Aliquots were taken for protein estimation using the Bio-Rad Coomassie blue assay and for the determination of [³H]thymidine incorporation (4). Data are expressed as cpm/ μ g protein and are the means of determinations made on six or more individual flasks.

Autoradiography

Cells were cultured for 24 hr in the presence of [³H]thymidine (2 μ Ci/ml) and then processed for autoradiography exactly as previously described (7). The percentage of labeled cells was determined by counting five individual areas in three different culture flasks (500 cells/area). Data are expressed as the means of 15 determinations.

Light Microscopy

Phase-contrast photomicrographs were taken with a Nikon inverted optics microscope using Ilford Pan F film.

Electron Microscopy

Cultured ventricular cardiac myocytes were processed for transmission electron microscopy (TEM) exactly as described previously (19). Briefly, the cells were fixed with 4.0% glutaraldehyde in 0.1 M sodium cacodylate (1 hr), postfixed in either 1.0% osmium tetroxide or 1.0% osmium tetroxide and 0.8% potassium ferrocyanide in the same buffer, *en bloc* stained in 0.5% uranyl acetate, dehydrated in an ascending series of alcohols, infiltrated in Epon-Araldite epoxy resin, and heat polymerized. *En face* (with respect to the culture substratum) silver sections were cut with a diamond knife on a Reichert OMU-3 ultramicrotome, poststained with lead citrate, and examined in a Philips 300 transmission electron microscope at an operating voltage of 60 kV. Images were recorded on Kodak electron microscope film.

Chemicals and Growth Factors

[Methyl-³H]thymidine (sp act, 20 Ci/mmole) was purchased from New England Nuclear; epidermal growth factor and fibroblast growth factor from Collaborative Research; insulin-like growth factor-I from AmGen Biologicals; dimethyl sulfoxide from J. T. Baker; and di-butyryl cyclic adenosine 3':5'-monophosphate, 12-O-tetradecanoyl-phorbol-13-acetate, 4- α -phorbol, 4- β -phorbol, 1,2-dicapryloyl-rac-glycerol, and 1-oleoyl-2-acetyl-rac-glycerol from Sigma.

RESULTS

The effects of several growth factors on DNA synthesis and protein content in cultured adult rat ventricular cardiac muscle cells are shown in Table 1. Insulin-like growth factor-I, (IGF-I), epidermal growth factor (EGF), and fibroblast growth factor (FGF) were all observed to stimulate [³H]thymidine incorporation into DNA in a concentration-dependent manner. These growth factors had no effect on total cellular protein. Cyclic AMP (cAMP), previously observed to inhibit DNA synthesis in other cardiac muscle preparations (4, 6), also inhibited DNA synthesis in these cultured adult myocytes. By far the greatest stimulation in DNA synthesis that we have observed was with the tumor promoter phorbol ester TPA (approximately 250%) (Table 2). Fifty nanograms per milliliter was the optimal concentration, and the maximal stimulation was determined to occur 5 days after adding TPA to the cultured myocytes (Fig. 1). The vehicle for TPA, dimethyl sulfoxide (DMSO), was observed to have little effect on DNA synthesis (Table 2). TPA also substantially increased the protein content in these cultured myocytes. The maximal concentration for this effect was also observed to be 50 ng/ml. In addition, TPA increases cellular RNA content (data not shown). The nontumor promoting phorbol ester analogs α - and β -phorbol had no effect on either DNA synthesis or protein content (Table 2). Protein kinase C is thought to be the receptor for TPA and to mediate most of its biological effects (18, 23-26). The

TABLE 1
EFFECT OF VARIOUS GROWTH FACTORS ON DNA SYNTHESIS AND PROTEIN CONTENT IN CULTURES OF CARDIAC MUSCLE CELLS

Factor tested	[³ H]Thymidine incorporation (% of control) ^a	Protein content (% of control) ^b
None (control)	100	100
IGF-I (20 ng/ml)	180 \pm 11	98 \pm 6
IGF-I (10 ng/ml)	152 \pm 9	99 \pm 8
IGF-I (4 ng/ml)	144 \pm 14	102 \pm 7
IGF-I (2 ng/ml)	131 \pm 9	101 \pm 8
EGF (25 ng/ml)	182 \pm 7	101 \pm 6
EGF (10 ng/ml)	142 \pm 10	98 \pm 7
FGF (30 ng/ml)	155 \pm 11	100 \pm 6
FGF (10 ng/ml)	137 \pm 12	97 \pm 5
cAMP (10^{-3} M)	62 \pm 4	98 \pm 6
cAMP (10^{-4} M)	84 \pm 6	106 \pm 9

Note. Adult rat ventricular cardiac muscle cells were cultured for 7 days (Day 7 of culture to Day 14) in the presence of the indicated growth factor and 2 μ Ci/ml of [³H]thymidine. The media was changed after Days 7, 9, and 11 of culture and the indicated growth factors and [³H]thymidine were added to the fresh media. The values are the means \pm SE of six determinations.

^a Control [³H]thymidine incorporation was 622 cpm/ μ g protein.

^b Control protein content was 154 μ g/flask.

TABLE 2
EFFECT OF TPA, PHORBOL ESTERS AND DIACYLGLYCEROLS ON DNA SYNTHESIS AND PROTEIN CONTENT IN CULTURES OF CARDIAC MUSCLE CELLS

Compound tested	[³ H]Thymidine incorporation (% of control) ^a	Protein content (% of control) ^b
None (control)	100	100
DMSO (0.02%)	94 ± 7	97 ± 4
Ethanol (0.01%)	84 ± 4	91 ± 7
TPA (100 ng/ml)	188 ± 9	185 ± 9
TPA (50 ng/ml)	259 ± 11	212 ± 9
TPA (25 ng/ml)	194 ± 10	187 ± 11
TPA (10 ng/ml)	146 ± 9	122 ± 6
DAG-A ^c (50 µg/ml)	195 ± 12	194 ± 12
DAG-A ^c (25 µg/ml)	147 ± 8	175 ± 6
DAG-B ^d (50 µg/ml)	165 ± 12	144 ± 8
DAG-B ^d (25 µg/ml)	142 ± 6	139 ± 10
α-Phorbol (100 ng/ml)	97 ± 4	101 ± 4
β-Phorbol (100 ng/ml)	94 ± 7	98 ± 7

Note. Adult rat ventricular cardiac muscle cells were cultured for 5 days (Day 7 of culture to Day 12) in the absence or presence of the indicated compound and 2 µCi/ml of [³H]thymidine. The media was changed after Days 7, 9, and 11 of culture. The values are the means ± SE of six determinations. TPA was dissolved in 100% DMSO and added to the media in small aliquots. The final concentration of DMSO in the media was 0.02%. Diacylglycerols were dissolved in 50% ethanol. The final concentration of ethanol in the media was 0.01%.

^a Control was 755 cpm/µg protein.

^b Control protein content was 147 µg/flask.

^c 1-Oleoyl-2-acetylglycerol.

^d 1,2-Dicapryloylglycerol.

normal cellular effector of this enzyme is diacylglycerol (DAG) (18, 23-26). Therefore, we tested whether or not diacylglycerol would mimic the effect of TPA. Two different 1,2-diacylglycerols, 1-oleoyl-2-acetylglycerol and 1,2-dicapryloylglycerol were both observed to significantly stimulate DNA synthesis and increase cellular protein content (Table 2).

In order to determine if the increase in DNA synthesis was due to a stimulation of DNA synthesis in cells already synthesizing DNA or whether it was due to cells not previously replicating DNA being induced to enter the S phase of the cell cycle, we carried out an autoradiographic analysis. Cells were cultured with [³H]thymidine and processed for autoradiography. We found a positive correlation between the percentage stimulation of DNA synthesis and the percentage increase in the number of cells labeled with [³H]thymidine (Table 3).

TPA also profoundly altered the morphology of the cultured myocytes. Figure 2 demonstrates the morphological differences at the light microscope level between cells cultured with TPA and either the α or the β inac-

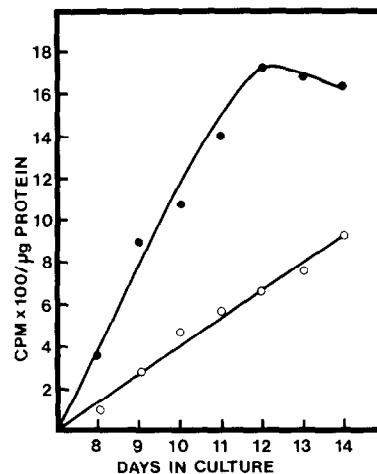


FIG. 1. Time course of TPA-stimulated DNA synthesis in cultured cardiac muscle cells. Adult rat ventricular cardiac muscle cells were cultured in the absence (○) or presence of 50 ng/ml TPA (●) from Day 7 to Day 14 of culture. TPA and [³H]thymidine (2 µCi/ml) were added with fresh media on Days 7, 9, 11, and 13 of culture. Values are the means of six determinations.

tive non-tumor-promoting phorbol ester analogs. Note that these micrographs are all at the same magnification. TPA-treated myocytes appear to have greatly enlarged in size (cell hypertrophy), but this change in apparent size may simply be due to a greater spreading of the cells on the surface of the culture flask. Multiple nuclei were observed in the cytoplasm of some of the TPA- and DAG-treated cells, and multiple or fragmented nucleoli were seen in some of the nuclei (micro-

TABLE 3
AUTORADIOGRAPHIC ANALYSIS OF DNA SYNTHESIS IN CULTURED CARDIAC MUSCLE CELLS

Compound tested	Cells labeled (% of control) ^a
None (control)	100
DMSO (0.02%)	94 ± 6
EGF (20 ng/ml)	144 ± 11
FGF (20 ng/ml)	156 ± 9
TPA (100 ng/ml)	161 ± 12
TPA (50 ng/ml)	244 ± 15
α-Phorbol (100 ng/ml)	94 ± 4
β-Phorbol (100 ng/ml)	89 ± 8

Note. Adult rat ventricular cardiac muscle cells were cultured for 5 days (Day 7 to Day 12 of culture) in the absence or presence of the indicated compound and 2 µCi/ml of [³H]thymidine and then were processed for autoradiography (7). The media was changed after Days 7, 9, 11, and 13 of culture. Values are the means ± SE of five individual determinations made on three different culture flasks.

^a Control labeling was 18% of total cell population.

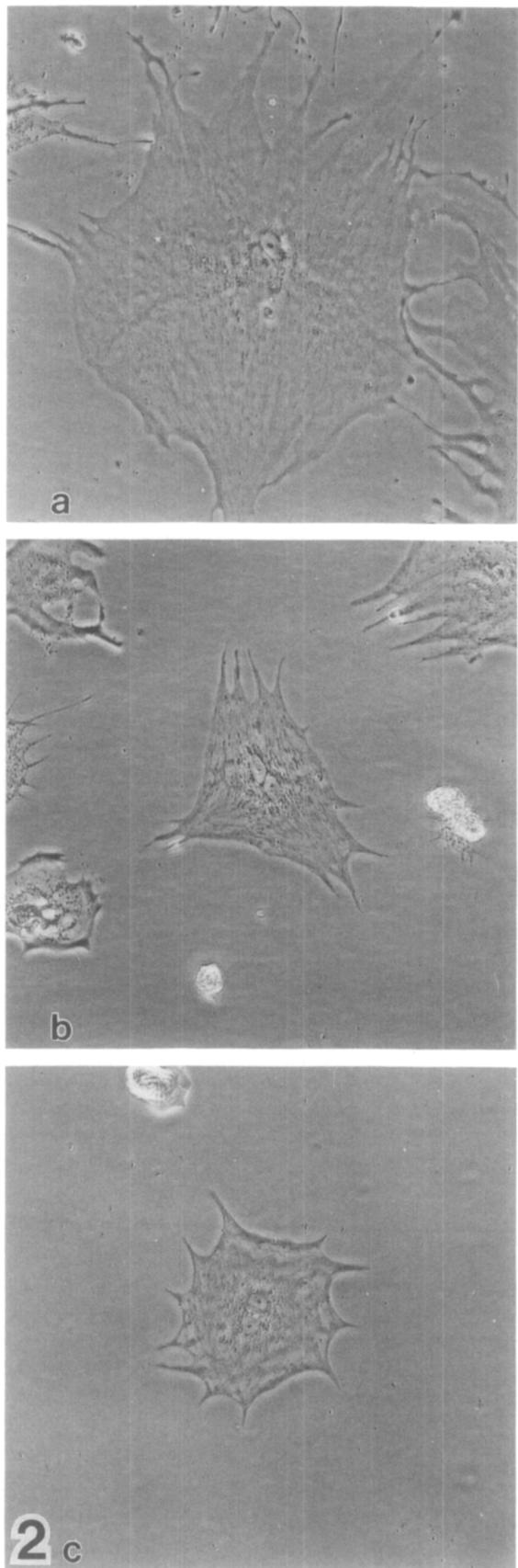


FIG. 2. Phase-contrast micrographs of cultured adult rat ventricular cardiac muscle cells treated with TPA (50 ng/ml) for 5 days (Day 7

graphs not shown). Most strikingly, TPA-treated myocytes were altered in overall morphology and lost their highly structured myofibrillar organization. Cells cultured in either DMSO or ethanol, the carrier for DAG, appeared similar to cells cultured with the inactive phorbol ester analogs, while myocytes cultured in the presence of DAG appeared similar to cells cultured with TPA. The growth factors tested had no observable effect detectable by either light or electron microscopy on cellular morphology.

The most striking ultrastructural effect of treatment of these cultured myocytes with either TPA (Fig. 4) or DAG (Fig. 3b) was the loss of typical myofibrillar morphology. Cells treated with either DMSO or ethanol (Fig. 3a) had ultrastructural characteristics identical to ventricular cardiac muscle cells cultured in their absence (see (9) and (19)). Myocyte ultrastructure was also unaffected after treatment with the inactive α - and β -phorbol analogs of TPA. Myocytes treated with DMSO, ethanol, or the TPA analogs had well-developed myofibrils and other ultrastructural features including a well-developed T system, internal and peripheral couplings, and intercalated discs typical of well-differentiated cultured ventricular cardiac muscle cells (Fig. 3a) (9, 19).

The ultrastructure of myocytes treated with either TPA or DAG was very similar. Well-organized myofibrils were rare in these cells. Both actin and myosin filaments were present, but their organization was ill-defined, and typical sarcomeres were rare. Irregular Z densities were often found in association with aggregations of thick and thin filaments. The location of these incomplete sarcomeres was inconsistent (Figs. 3b and 4). Leptomeres (21, 27) were found much more frequently in the TPA- and DAG-treated myocytes than in any of the control groups. These were most frequently observed near the myocyte periphery and consisted of alternating Z lines and collections of actin filaments (Fig. 3b).

Although cells treated with TPA and DAG responded uniformly with respect to myofibrillar ultrastructure, other myocyte components responded somewhat variably. Some of the myocytes responded by accumulating large amounts of intermediate filaments or large amounts of rough endoplasmic reticulum and free ribosomes (Fig. 4). Occasionally, dense-cored, membrane-bound vesicles were observed, but these were much scarcer than those observed in cultured adult atrial myocytes (20). The TPA- and DAG-treated myocytes

of culture to Day 12) (a) or with the nontumor promoter phorbol ester α (b) or β (c) TPA analogs (100 ng/ml). The chemicals were added at Day 7 and the media was changed after Days 9 and 11 of culture. Note the much larger dimensions of the TPA-treated cell. All micrographs, $\times 190$.

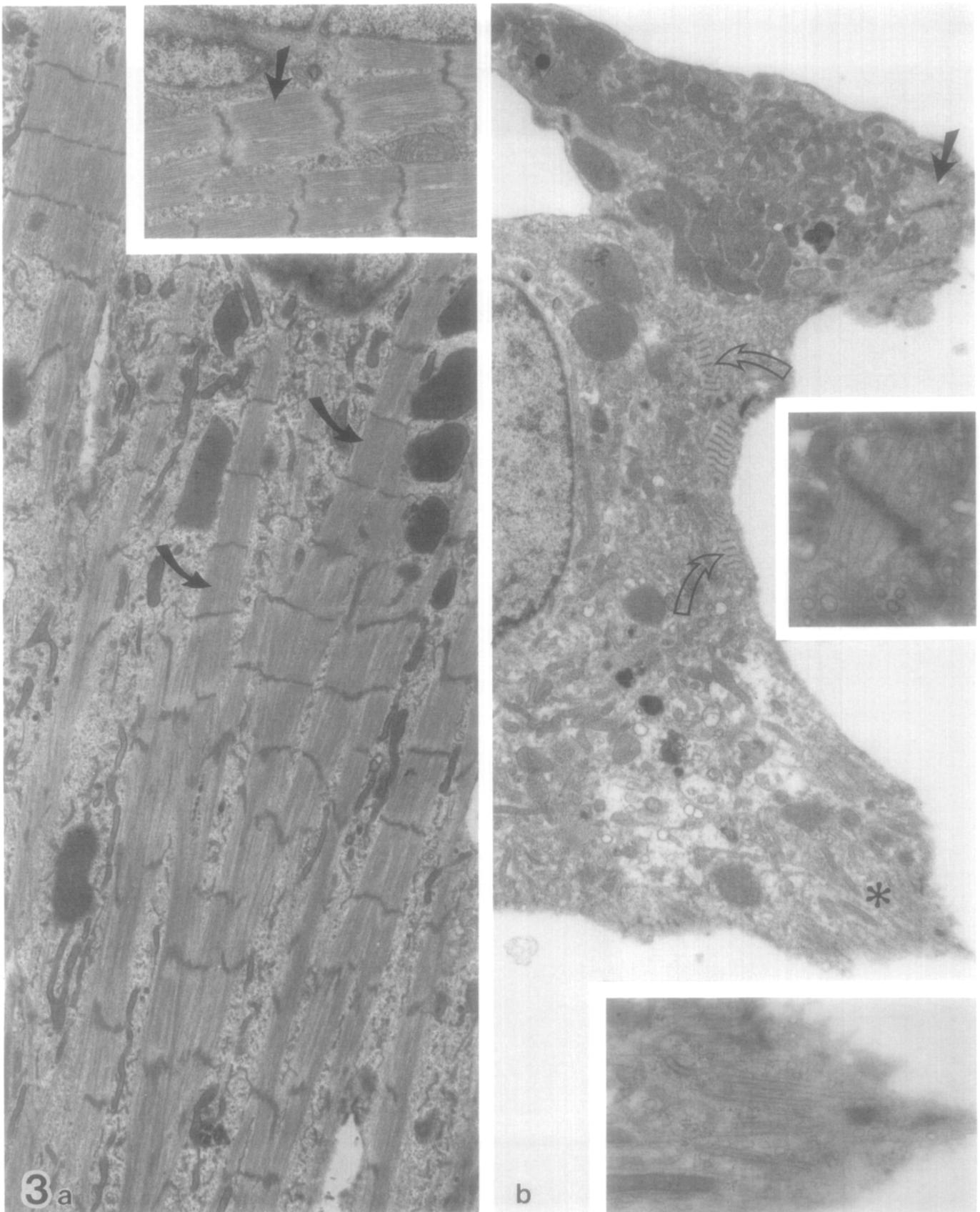
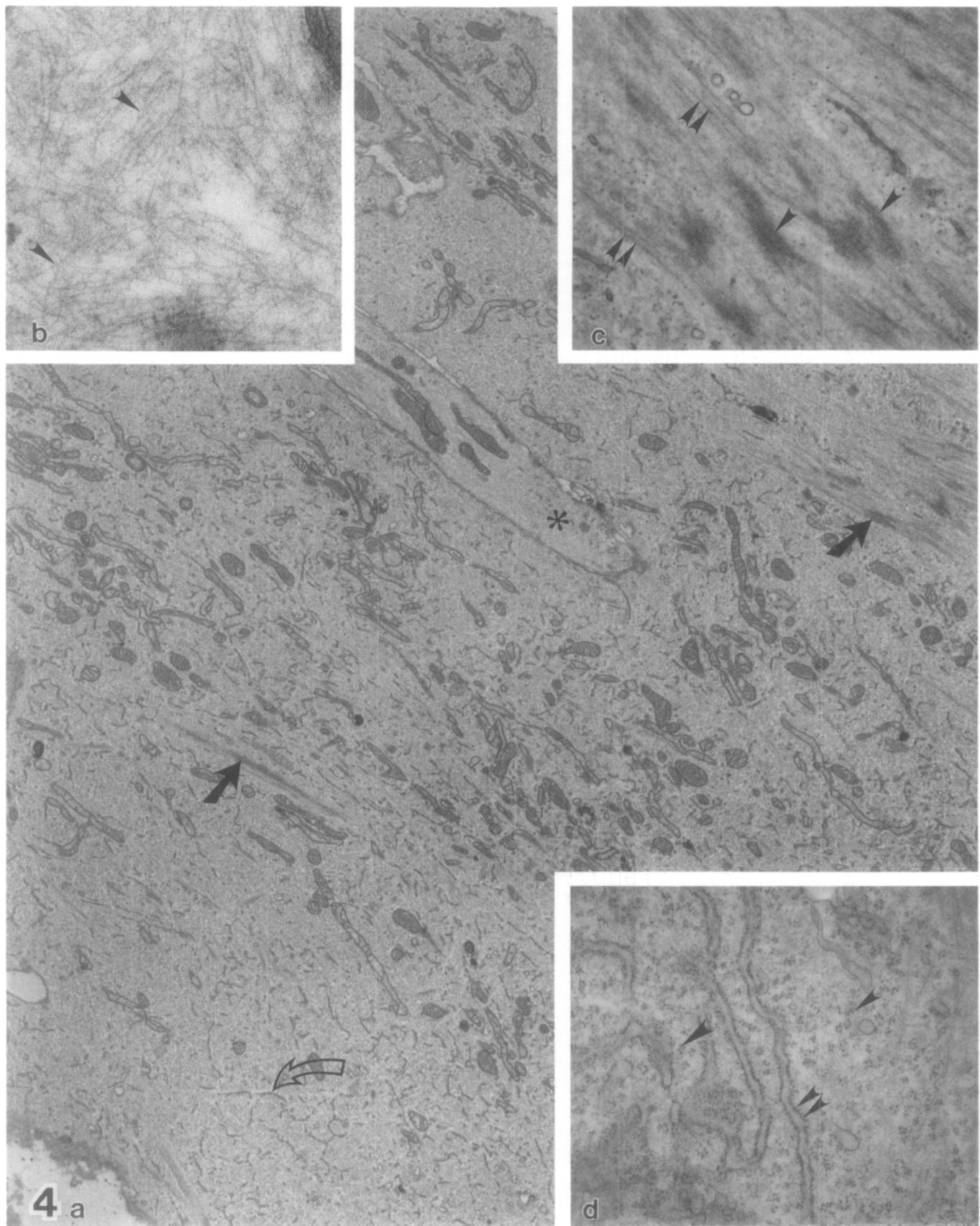


FIG. 3. (a) Electron micrograph of DMSO-treated (final concentration of DMSO was 0.02%) cultured adult cardiac ventricular myocyte. Note the well-developed sarcomeres (arrows). Inset, similar electron micrograph of an ethanol-treated (final concentration was 0.01%) myocyte. Again note the well-developed sarcomeres (arrow). (a) $\times 12,000$. Inset, $\times 13,900$. Treatment was exactly the same as that specified in Fig. 2. (b) Electron micrograph of DAG-treated (50 $\mu\text{g}/\text{ml}$) adult ventricular myocyte. Note that the sarcomeres (filled arrow and upper inset) lack myofibrillar organization. Leptomeres (open arrows) are present. The sarcomere, marked by the filled arrow, is shown at higher magnification in the upper inset. Although a Z line and both actin and myosin filaments are present, sarcomeric organization is incomplete. The area marked by the * is shown at higher magnification in the lower inset. Note the disorganized actin and myosin filaments. Treatment was exactly the same as that specified in Fig. 2. (b) $\times 9,200$. Insets, $\times 27,750$. All micrographs are of cells postfixed in osmium tetroxide.



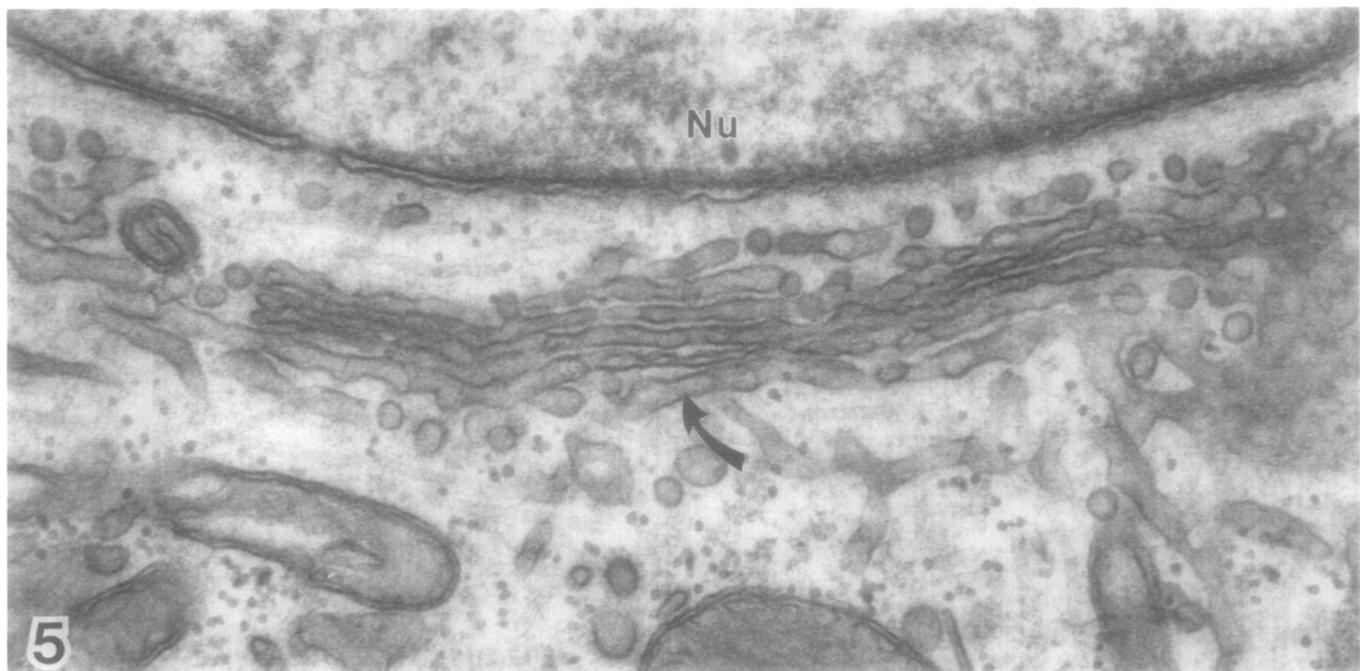


FIG. 5. Electron micrograph of perinuclear region of TPA-treated (treatment was exactly the same as that specified in Fig. 2) ventricular cardiac myocyte (osmium ferrocyanide postfixation). Note the well-developed Golgi apparatus. Nu, nucleus; arrow, Golgi lamellae. $\times 74,000$.

had well-developed Golgi complexes similar to those of the control cells (Fig. 5) (19, 20).

Overall, the ultrastructure of TPA- and DAG-treated ventricular myocytes was much less organized than cells in any of the control classes. The presence of the highly organized structures characteristic of well-differentiated cardiac muscle cells was greatly reduced or totally absent.

DISCUSSION

These studies demonstrate that DNA synthesis in cultured terminally differentiated adult rat ventricular cardiac muscle cells can be stimulated by several growth factors, the tumor promoter TPA, and DAG. Moreover, there are distinct ultrastructural alterations after treatment with TPA or DAG. TPA and DAG also substantially increase cellular protein content. Although we have not, as yet, definitively identified which

cellular proteins accumulate, our preliminary experiments indicate that TPA greatly increases the content of intermediate filaments. This is in agreement with what was observed when cultured skeletal muscle cells were treated with TPA (1, 12-14, 16, 18a, 30). Our ultrastructural studies indicate that some of this cellular protein accumulation is due to the synthesis of new ribosomes (Fig. 4).

Apparently, stimulation of DNA synthesis by growth factors, and by TPA and DAG, occurs by different mechanisms. Tyrosine-specific protein kinases mediate the biological response of several growth factors. This has been established for IGF-I, EGF, and FGF whose cellular receptors have been shown to have tyrosine kinase activity (2, 17, 28, 29). Most of the effects of TPA and DAG are known to be mediated by a serine/threonine-specific, calcium/phospholipid-dependent protein kinase (protein kinase C) (18, 23-26). Our studies suggest that DNA synthesis in these terminally differen-

FIG. 4. (a) Low power electron micrograph of TPA-treated (treatment was exactly the same as that specified in Fig. 2) adult ventricular cardiac muscle cell. Note the lack of well-defined sarcomeres. Sarcomeric organization is limited to Z densities with loosely associated collections of actin and myosin filaments (arrows). A similar area is shown at higher magnification in (c). The * indicates a cell which has accumulated numerous intermediate filaments. A higher magnification of a similar cell is shown in (b). The open arrow indicates an area of the cell which has accumulated quantities of rough endoplasmic reticulum. A similar area is shown at higher magnification in (d). $\times 11,250$. (b) Higher magnification of an area similar to that marked by * in (a). The cytoplasm is largely given over to intermediate filaments (arrowheads). $\times 71,000$. (c) Higher magnification of an area similar to that marked by the arrows in (a). Note the irregular Z lines (arrowheads) and thick myosin filaments (double arrowheads). $\times 27,750$. (d) Higher magnification of an area similar to that marked by the open arrow in (a). Note that both free (arrowhead) and attached (double arrowhead) ribosomes are present. $\times 27,750$. (a) and (c) are postfixed in osmium ferrocyanide. (b) and (d) are postfixed in osmium tetroxide.

tiated cardiac muscle cells may be stimulated by different mechanisms. This concept is supported by our observations on the structural alterations induced by TPA and DAG, but not by growth factors.

DNA synthesis is reactivated in these terminally differentiated cardiac myocytes when they are grown in primary culture (7), an event that occurs in the absence of exogenously added growth factors or agents such as TPA and DAG. Previously this capacity was believed to be permanently lost during early neonatal development (6). Growth factors and/or other substances in the serum in which these cells are normally cultured may be causative factors in reactivating this DNA synthetic activity. This reactivation could also be due simply to a loss of contact inhibition when the cells are isolated from the intact muscle.

The stimulation of DNA synthesis and alteration in cellular morphology by TPA and DAG suggests a possible structure-function relationship, although these two events may not be causally related. TPA induces an almost total loss of myofibrillar ultrastructure in these highly differentiated muscle cells. It appears to induce these cells to revert back to a less differentiated state in which they are less organized structurally and are actively synthesizing DNA. TPA may reactivate the embryonic developmental program of these cardiac muscle cells and inactivate the terminal differentiation program. Preliminary work indicates that TPA almost totally represses the expression of myosin heavy chain and muscle-specific creatine kinase genes in these cells, and activates the expression of several proto-oncogenes whose expression is only observed in neonatal cardiac myocytes ((6a, 11) and Claycomb, unpublished observation).

Holtzer and colleagues (1, 12-14, 16, 18a, 30) have carried out extensive studies on the effect of TPA on skeletal muscle differentiation and have used this paradigm as a model to investigate the involvement of the cytoskeleton in myofibrillogenesis. Their studies have shown that TPA essentially transforms cultured skeletal muscle cells and that many of the morphological changes observed with TPA mimic those seen in skeletal muscle cells infected with Rous sarcoma virus. These TPA-treated skeletal muscle cells, in common with our TPA-treated cardiac myocytes, lose their highly organized myofibrillar ultrastructure and accumulate intermediate filaments. At the biochemical level, these investigators have observed that TPA inhibited the synthesis and stimulated the degradation of myotube-specific proteins such as myosin, α -actin, troponin C, and tropomyosin. They have also shown that not all muscle-specific proteins are produced in reduced quantities. For instance, desmin, a muscle-specific intermediate filament protein, is produced in normal quantities

but not degraded as rapidly (30). This is in agreement with our ultrastructural data showing that intermediate filaments accumulate in TPA- and DAG-treated cells. In contrast to our results, these investigators report that TPA had no effect on DNA synthesis in skeletal muscle cells (30). They have also recently reported that TPA did not block the contraction of beating chick cardiac myocytes nor did it disrupt the integrity of their myofibrils (18a). It may be that there are major differences in the effect of TPA on chick and mammalian cardiac muscle cells. It is also possible that the TPA response is dependent on the degree of differentiation of the cell to which it is applied (embryonic vs terminally differentiated).

TPA is also capable of affecting the morphology and cytoskeletal architecture of a number of other cell types. Many epithelial cells respond to TPA treatment by accumulating intermediate filaments, and this response has been suggested to be an ultrastructure "signature" of cellular transformation (15).

The other common response that we observed in TPA- and DAG-treated myocytes, the accumulation of rough endoplasmic reticulum and free ribosomes (Fig. 4), has not been reported by other workers. The free ribosomes are probably prominently involved in the synthesis of intermediate filaments' proteins, as well as numerous other protein species. The presence of well-developed Golgi complexes (Fig. 5) is indicative of potential secretory activity. The proteins synthesized on the rough endoplasmic reticulum are more problematical. Secretory granules such as those observed in cultured adult atrial myocytes (20) were rare in both control and experimental ventricular myocytes. Presumably, proteins manufactured on membrane-bound ribosomes are destined for export, and future studies will address their identity and disposition.

In summary, this TPA-induced cellular plasticity should be very useful for studies of the regulation of cardiac muscle cell differentiation and cell proliferation and could lead to an understanding of how DNA synthesis is permanently repressed *in vivo* during the early development of the mammalian heart.

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