

Sequential Disassembly of Myofibrils Induced by Myristate Acetate in Cultured Myotubes

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Abstract. The phorbol ester TPA induces the sequential disassembly of myofibrils. First the alpha-actin thin filaments are disrupted and then, hours later, the myosin heavy chain (MHC) thick filaments. TPA does not induce the disassembly of the beta- and gamma-actin thin filaments of stress fibers in presumptive myoblasts or fibroblasts, nor does it block the reemergence of stress fibers in 72-h myosacs that have been depleted of all myofibrillar molecules.

There are differences in where, when, and how myofibrillar alpha-actin and MHC are degraded and eliminated from TPA-myosacs. Though the anisodiametric myotubes have begun to retract into isodiametric myosacs after 5 h in TPA, staining with anti-MHC reveals normal tandem A bands. In contrast, staining with mAb to muscle actin fails to reveal tandem I bands. Instead, both mAb to muscle actin and rho-phalloidin brilliantly stain numerous disk-like bodies ~3.0 µm in diameter. These muscle actin bodies do

not fuse with one another, nor do they costain with anti-MHC. All muscle actin bodies and/or molecules disappear in 36-h myosacs. The collapse of A bands is first initiated in 10-h myosacs. Their loss correlates with the appearance of immense, amorphous MHC patches. MHC patches range from a few micrometers to over 60 µm in size. They do not costain with antimuscle actin or rho-phalloidin. While diminishing in number and fluorescence intensity, MHC aggregates are present in 30% of the 72-h myosacs. Myosacs removed from TPA rapidly elongate, and after 48 h display normal newly assembled myofibrils. TPA reversibly blocks incorporation of [³⁵S]methionine into myofibrillar alpha-actin, MHC, myosin light chains 1 and 2, the tropomyosins, and troponin C. It does not block the synthesis of beta- or gamma-actins, the non-myofibrillar MHC or light chains, tubulin, vimentin, desmin, or most household molecules.

12 -O-TETRADECANOYL phorbol-13-acetate (TPA) binds to protein kinase C, resulting in full activation at physiological concentrations of Ca²⁺. Protein kinase C is present in many types of cells. It has a broad substrate specificity, phosphorylating seryl and threonyl residues in many proteins. TPA influences many major metabolic pathways directly by mobilizing protein kinase C and indirectly by altering the levels of inositol (1,4,5)-triphosphate and the activity of both the K⁺ channels and the Na⁺/H⁺ exchanger (see references in 3, 7, 35, 45, 48).

That the pleiotropic effects of TPA depend on the ongoing differentiation program of the responding cell is illustrated by its selective, but reversible, effects on cells in different compartments of the myogenic lineage. TPA acts as a mitogen for replicating presumptive myoblasts, inducing them to form long processes and form multilayers of "fibroblastic" cells. These cells have the properties expected of cells arrested in the penultimate compartment of the myogenic lineage (25, 31, 64). Within minutes TPA also blocks the fusion of postmitotic mononucleated myoblasts into multinucleated

myotubes (6, 8, 29, 30). TPA has still other effects on postmitotic multinucleated myotubes. It rapidly, but selectively, disassembles the striated myofibrils in anisodiametric myotubes and converts them into large, isodiametric multinucleated myosacs (2, 11, 12, 14, 58). The selective depletion of assembled myofibrils and the conversion of myotubes into myosacs is not due to a generalized cytotoxic response, as total protein synthesis is inhibited only 20–25%, or that fraction of total protein synthesis normally accounted for by the myofibrillar proteins in cultured myotubes (5). These catabolic effects of TPA on myotubes are reversible. In normal medium, the isodiametric myosacs will elongate and within 48 h assemble a complement of myofibrils that requires normal myotubes 5–6 d to assemble (11, 14, 58).

In the following experiments TPA is used to probe aspects of myofibril and stress fiber assembly and disassembly. These experiments are not designed to localize which of the multiple targets affected by TPA is responsible for the reversible blocking of myofibrillogenesis. Rather, TPA is used as an agent to probe the response of the contractile proteins in

muscle and nonmuscle cells, such as Colcemid or vinblastine is used to probe the response of cells whose microtubules (MTs)¹ have been depolymerized. By using TPA as a reversible perturbing agent, we found that: (a) TPA does not disassemble the beta- and gamma-actin thin filaments of stress fibers in primary replicating presumptive myoblasts or fibroblasts, nor does it block the de novo reassembly of stress fibers in spreading TPA-myosacs; (b) the I-Z-I complex of myofibrils begins to disappear in myotubes exposed to TPA for 3–5 h; (c) this induced resorption of the I-Z-I complex correlates with the emergence of hundreds of 3.0- μ m muscle-actin “bagels” and, subsequently, muscle-actin disks; (d) only hours later do the tandem A bands collapse into immense, irregular myofibrillar myosin heavy chain (MHC) patches; and (e) the phorbol ester selectively, but reversibly, blocks the synthesis of most (all?) myofibrillar isoforms, but spares the synthesis of the nonmyofibrillar myosin heavy and light chains, and the beta- and gamma-actins and tropomyosins, as well as that of desmin, vimentin, and tubulin.

Materials and Methods

Cell Culture

Primary cultures of mononucleated cells were prepared from breast muscles of 11-d chick embryos (1, 5). Day 4 cultures were exposed to TPA (kindly provided by Dr. T. G. O'Brien, Wistar Institute, Philadelphia, PA) at a concentration of 75 ng/ml for periods of 1 to 72 h (11, 12). Selected control cultures were treated with the inactive phorbol ester 4 alpha-phorbol-12,13-didecanoate, which had no obvious effect on these cultured cells. Control and TPA-treated cultures were fed daily. Cycloheximide (final concentration 0.1 mM; Sigma Chemical Co., St. Louis, MO) was added to medium immediately before addition to cultures. At this concentration protein synthesis in myogenic cultures is inhibited over 95% (10, 18).

Antibodies

Two different antibodies have been used to localize skeletal muscle MHC, a rabbit polyclonal against chicken light meromyosin (anti-LMM), and a mouse monoclonal against chicken MHC (a gift of Dr. F. Pepe, University of Pennsylvania). Both antibodies stain A bands in developing muscle but do not stain the myosin in presumptive myoblasts, fibroblasts, smooth muscle, or other cell types examined. In immunoblots both antibodies stain skeletal MHC, but do not stain MHC from smooth muscle or nonmuscle cells. These two antibodies have been used interchangeably and are referred to as anti-MHC. Antibodies against smooth muscle alpha-actinin and against chick brain myosin were generous gifts from Dr. S. Craig (Johns Hopkins University, Baltimore, MD) and Dr. V. Nachmias (University of Pennsylvania), respectively. The properties of these antibodies with respect to cultured myogenic cells have recently been described (1, 2, 15, 24). Antitubulin was purchased from Miles Laboratories Inc., Naperville, IL. To follow the presence or absence of alpha- or muscle-actin, a mouse monoclonal antibody against chicken gizzard actin was used (mAb B4). This antibody binds to all four known muscle actins (alpha-skeletal, alpha-cardiac, alpha-vascular, and gamma-enteric actin) on immunoblots and using indirect immunofluorescence on methanol-fixed tissues. mAb B4 does not react with either beta- or gamma-cytoplasmic actin from nonmuscle cells on immunoblots or indirect immunofluorescence (Lessard, J. L., manuscript submitted for publication).

Immunofluorescence Staining

Processing cells for immunofluorescence microscopy has been described in detail (1, 15, 16). Various sequences of antibody or reagent incubations were used to produce the double-stained preparations. Monoclonal anti-MHC was visualized by indirect immunofluorescence, whereas FITC-labeled anti-

1. *Abbreviations used in this paper:* EMS, ethyl-methylsulfonate; MHC, myosin heavy chain; MTs, microtubules; rho-phalloidin, rhodamine-labeled phalloidin.

LMM was visualized directly. A previously described procedure (53) was used to stain the same cells with anti-LMM and another rabbit antibody. After the primary antibody incubation, cells were washed three times, incubated with secondary antibody (rhodamine-labeled goat anti-rabbit IgG, Cooper Laboratories, Malvern, PA), and washed again. Before anti-LMM was applied, the cells were incubated for 15–20 min at room temperature in a 1:5 dilution of normal rabbit serum in PBS to saturate exposed goat anti-rabbit IgG sites. Cells were then incubated with anti-LMM, washed, and mounted using 60% glycerol/PBS. Rhodamine-labeled phalloidin (rhodalloidin), which binds specifically to F-actin (62), was used to visualize stress fibers and thin filaments within myofibrils. Antitubulin staining for MTs was performed as described (18). In some preparations, the DNA-binding fluorochrome, Hoechst 33342, or 4,6-Diamino-2-phenylindole (DAPI) was used to stain nuclei. Cells were examined with a Zeiss epifluorescence microscope using excitation filters for either fluorescein or rhodamine fluorescence and a short band pass barrier filter for fluorescein that eliminates most bleed-through between channels.

[³⁵S]Methionine Labeling of Newly Synthesized Proteins

Protein synthesis levels were detected by washing cultures in balanced salt solution (BSS) and incubating them in 2 ml of methionine-free medium (Flow Laboratories, McLean, VA) containing 25 μ Ci/ml [³⁵S]methionine (1,200–1,500 Ci/mmol; Amersham Corp., Arlington Heights, IL). Cultures were labeled for 60 min at 37°C. For protein accumulation, control or treated cultures were fed daily with appropriate media containing 15 μ Ci [³⁵S]methionine/ml of medium, during the 48 h before sacrifice.

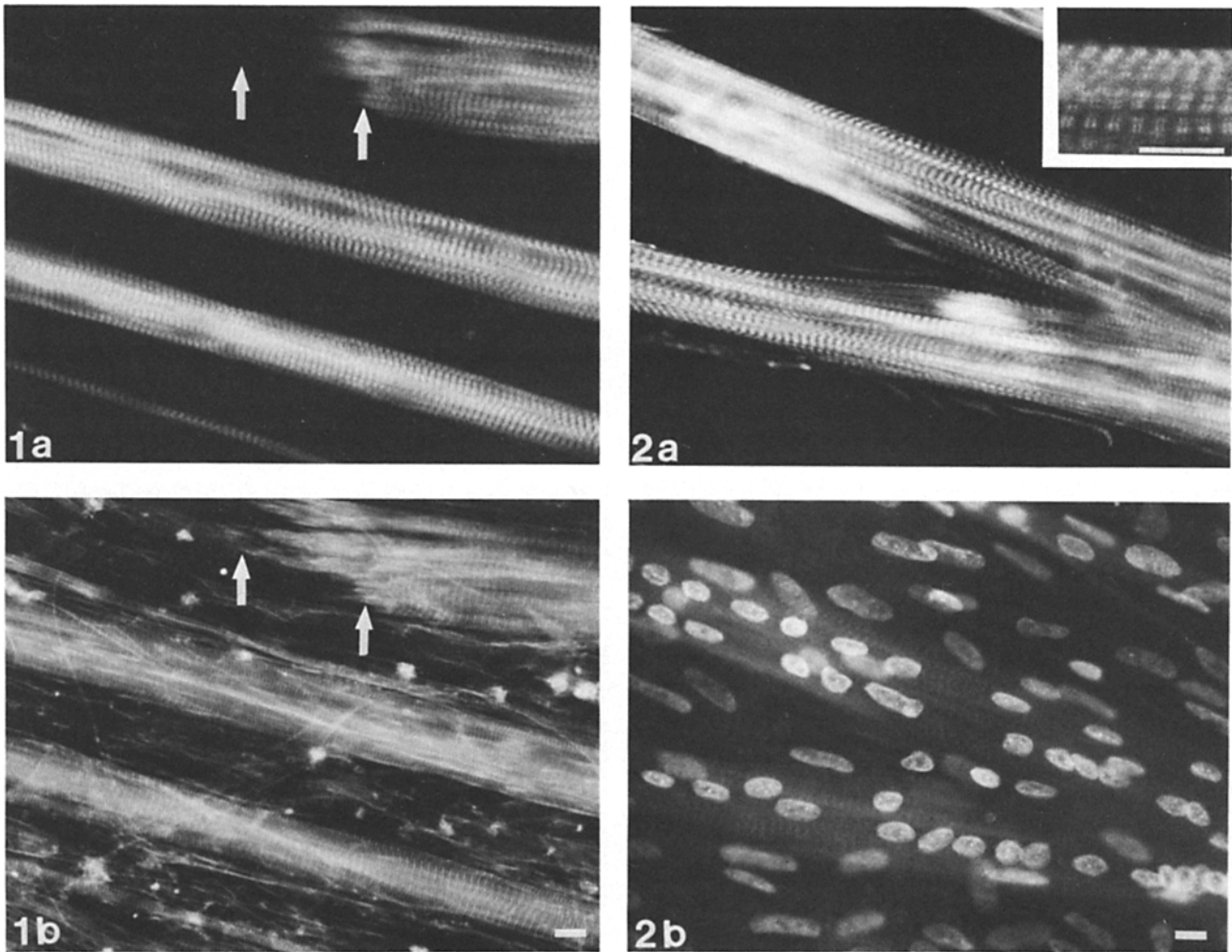
Samples were prepared on ice in the presence of proteolysis inhibitors (5 mM EDTA, 5 nM benzamide, 5 mg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). Whole cell samples were prepared by scraping cells into PBS containing proteolysis inhibitors, sonicating, and precipitating them with acetone, and solubilizing the resultant pellets in either Laemmli sample buffer (38) or lysis buffer. Total protein in Triton-soluble and -insoluble portions was determined by a microassay procedure (Bio-Rad Laboratories, Richmond, CA) using BSA as a standard. For determination of radioactivity in individual samples, aliquots were removed, placed in hydrofluor scintillation cocktail (National Diagnostics Inc., Sommerville, NJ) and counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Unlabeled and labeled proteins were separated for isoelectric focusing two-dimensional SDS-PAGE and immunoblots as described elsewhere (1, 2, 28). The radioactivity in individual proteins of samples containing an equal number of counts per minute was determined by excising spots or bands from dried gels. Gel pieces were incubated overnight at room temperature in scintillation cocktail (88% vol/vol toluene, 10% vol/vol NCS tissue solubilizer, 2% vol/vol H₂O, 0.6% wt/vol PPO, 0.015% wt/vol dimethyl PPO). Background radioactivity was determined by excising portions of each gel that lacked protein; these values were subtracted from total radioactivity in each protein. Chicken breast muscle myosin standard (Sigma Chemical Co.) and a glycerinated myofibril preparation were used to identify myosin heavy and light chains, alpha-actin, alpha-tropomyosin, and troponin C. Twice-cycled calf brain microtubules were used as a tubulin standard. Bound antibody was visualized by peroxidase-antiperoxidase. Nitrocellulose was autoradiographed to visualize all proteins that had been transferred.

Results

Mononucleated Cells and Myotubes in Day 4 Myogenic Cultures

Day 4–5 cultures contain 2–3 $\times 10^6$ replicating, fibroblastic cells. Roughly 80% are presumptive myoblasts, 20% fibroblasts (5, 10, 13, 31, 64). The majority of spread mononucleated fibroblastic cells display prominent stress fibers (Figs. 5 a, 8 a, and 9 a) that stain uniformly with rhodalloidin, but bind antibodies to chick brain (CB)-myosin and alpha-actinin in a punctate fashion (see figures in references 1 and 15). Only very rarely do fibroblastic cells bind mAb B4 (Figs. 3 b, 6 a and b, and 7) or the myofibrillar anti-MHC (Figs. 4 b, 5 b, 8 b, and 9 b).



Figures 1–2. (Fig. 1, *a* and *b*) Micrographs of a day 4 myogenic culture double-stained with anti-MHC (*a*, fluorescein channel) and rho-phalloidin (*b*). Anti-MHC localizes to the definitive A bands in the body of the myotube. The MHC-positive, nonstriated, distal ends of the myofibrils (*right arrow*) extend for short distances into the base of the myotube's growth tip (*left arrow*). The anti-MHC does not stain the numerous mononucleated presumptive myoblasts or fibroblasts that lie between, and atop, the anisodiametric myotubes. Rho-phalloidin stains the same myofibrils in a complex sarcomeric pattern of broad and narrow transverse bands (*b*). Their number and width varies with contraction. The rho-phalloidin nonstriated, distal ends of the myofibrils (*right arrow*) are continuous with the packed stress fibers that extend for considerable distances into the myotube's growth tip (*left arrow*). The rho-phalloidin-positive stress fibers in the mononucleated cells are poorly visualized in this particular micrograph because they are not in the plane of focus (see Figs. 5 *a*, 8 *a*, and 9 *a*). Note the absence of long stress fibers in the body of these myotubes. Bar, 10 μm . (Fig. 2, *a* and *b*) Micrographs of a day 4 control culture double-stained with muscle-actin mAb B4 (*a*, rhodamine channel) and Hoechst 33342 (*b*, blue channel). The anti-muscle actin binds to the I bands; as illustrated in the inset, it does not stain the Z bands. The high density of the surrounding mononucleated cells can be appreciated by examining *b*, which shows the nuclei in this field. The anti-muscle actin does not stain the prominent stress fibers that are present in the spread fibroblastic, mononucleated cells distributed between the anisodiametric myotubes. Bar, 10 μm .

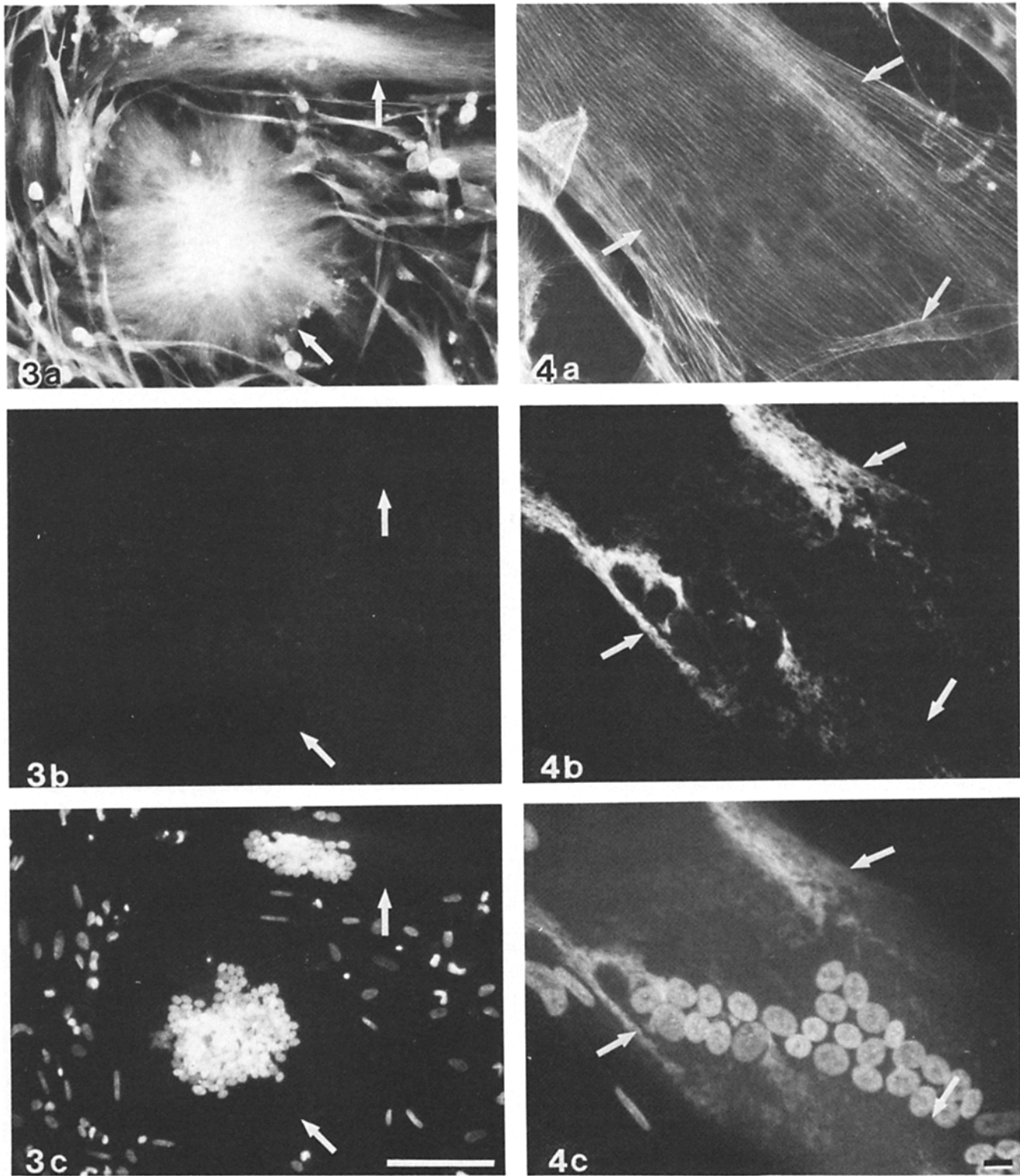
Day 4–5 cultures also contain thousands of multinucleated, anisodiametric myotubes. A single myotube averages 10–15 μm in diameter and can be over 3,000 μm in length. All myotubes display large numbers of striated myofibrils consisting of definitive A, I, Z, and M bands. They contract spontaneously. Antibodies to MHC stain A bands (Fig. 1 *a*), whereas mAb B4 to muscle actin stains I bands (Fig. 2 *a*). As recently reported (1, 15, 28), rho-phalloidin stains myofibrils in a distinctive, though complex, sarcomeric pattern that depends on degree of contraction (Fig. 1 *b*).

Subcortical stress fibers course throughout the body of normal day 2 and 3 myotubes. Their number declines markedly during day 4. They largely disappear from the body of

day 5 myotubes, remaining only in the growth tips (Fig. 1 *b*). The stress fibers in myotubes stain as do the stress fibers in mononucleated cells, i.e., uniformly with rho-phalloidin and in a nonsarcomeric, punctate fashion with antibodies to CB-myosin and alpha-actinin (data not shown).

TPA Disassembles Myofibrils but Sparing Stress Fibers and MTs

Spontaneous contractions cease 1 h after TPA is added to day 4 cultures. At this time the anisodiametric myotubes begin to flatten and retract slowly. By 5 h their smooth profile is broken by lateral, sawtooth, emerging pseudopodial processes (Fig. 6 *a* and *b*). These processes display prominent stress



Figures 3–4. (Fig. 3, *a–c*) Low power micrographs of a microscopic field providing an overview of the surrounding mononucleated cells and two multinucleated 36-h myosacs (day 4 myotubes cultured for an additional 36-h in TPA). This culture has been stained with antitubulin (*a*, fluorescein channel), anti-muscle actin (*b*, rhodamine channel), and DAPI (*c*, blue channel). Note in *a* the enormous number and radial arrangement of the long microtubules in the medallion-shaped myosac (*lower arrow*) vs. their more longitudinal distribution in the still somewhat elongated myosac (*upper arrow*). All the mononucleated cells also bind the antitubulin. *b* demonstrates that not only have the myofibrillar thin filaments that had been present 36 h earlier been totally disassembled, but that all molecules that bind mAb B4 have been cleared from these myosacs. *c* illustrates the characteristic clustering of nuclei toward the center of the isodiametric, multinucleated myosacs. Bar, 100 μ m. (Fig. 4, *a–c*) A triple-stained 72-h myosac selected for its persistent MHC-positive amorphous patches (*b*, fluorescein channel), and its long, parallel rho-phalloidin-positive stress fibers (*a*). The stress fibers in such flattened myosacs are negative for mAb B4. Note that the rho-phalloidin does not costain with the MHC aggregates. The mAb B4 also does not localize to the MHC patches (data

fibers and numerous MTs. The stress fibers and MTs of these processes are oriented at right angles to the long axis of the retracting myotube and often display ruffled membranes. These changes continue so that after 48–72 h in TPA, the originally elongated myotubes have gradually transformed into flattened, often isodiametric, myosacs. Accompanying this change in overall morphology is a change in distribution of nuclei from a row in the middle of the myotube to a cluster(s) in the center of the myosac (Figs. 3 *c* and 4 *c*).

During this period of transformation from myotube to myosac, the alpha-actin thin filaments and the MHC thick filaments are sequentially disassembled and degraded. Nothing remotely resembling structured myofibrils can be detected in 24-h myosacs (i.e., day 4 cultures exposed to TPA for an additional 24 h) stained with either mAb B4 or anti-MHC. Virtually all muscle actin molecules have been cleared from 24–36-h myosacs (Fig. 3 *b*). The clearing of all MHC molecules lags behind considerably, but roughly 70% of the myosacs are also totally negative for MHC molecules by 72 h. On the other hand, even as myofibrils are gradually disassembled, elsewhere in the same myosac new stress fibers are gradually assembled (see below). This differential response of myofibrils and stress fibers in myosacs is consistent with the observation that the stress fibers in the mononucleated cells in these TPA-treated cultures are not noticeably altered (Figs. 5 *a*, 8 *a*, and 9 *a*). In brief, although TPA dismantles I-Z-I complexes rapidly and the thick filaments of myofibrils more slowly, it has no obvious effect on the stress fibers in mononucleated cells that consist of beta- and gamma-actins, CB-myosin, alpha-actinin, etc., nor does it block the de novo reassembly of stress fibers in myofibril-depleted spreading myosacs.

Isodiametric myosacs of the kind illustrated in Fig. 3 rapidly recover when shifted to normal medium. Within 24 h they reacquire their anisodiametric morphology and reinitiate the assembly of new striated myofibrils. At this point in recovery they again lose their stress fibers. By 48 h the recovered myosacs are difficult to distinguish from untreated day 5–6 controls (11, 12, 28, 58). If reexposed to TPA, these recovered myotubes again: (*a*) resorb their striated myofibrils, (*b*) retract to form myosacs, and (*c*) reassemble prominent stress fibers. These second generation myosacs recover when TPA is removed from the medium; in the process of recovery, they again lose their stress fibers as they assemble a third generation of myofibrils.

The following sections detail the sequence of events associated with the transformation of a myotube full of myofibrils into a myosac depleted of most (all?) myofibrillar proteins, but rich in newly assembled stress fibers.

Waxing and Waning of Muscle Actin Bagels and Disks in TPA-Myosacs

After 3 h in TPA ~20% of the myotubes double-stained with mAb B4 and rho-phalloidin reveal not only striated myofibrils, but also many actin-positive bodies. These muscle ac-

tin bodies are roughly 3.0 μm in diameter (Figs. 5 *a*, 6, *a* and *b*, 7, 8 *a*, and 9 *a*). They do not costain with anti-MHC (Figs. 5 *b*, 8 *b*, and 9 *b*). As the core of these first-appearing circular bodies does not stain with either mAb B4 or rho-phalloidin, they will be referred to as muscle actin “bagels.” Muscle actin bagels are observed only in myosacs. They are never observed in the adjacent mononucleated fibroblastic cells.

There is considerable asynchrony in the appearance of actin bagels, as well as in their total number and density per myosac. Present in roughly 20% of the 3-h myosacs, they increase to 80% in 5-h myosacs. During this period there is a shift from actin bagels to actin disks (Table I). It is not known whether the nonstaining core of the actin bagel is due to failure of the reagents to penetrate or to the presence of molecules other than actin. Within a single myosac there can be stretches lacking actin bodies, alternating with stretches in which they are present. Most commonly, they accumulate parallel to, and in proximity to the undersurface of the myosac. Rarely do they abut one another in the center of the still-elongated myosac. More peripherally, however, they can form strings of up to 10 contiguous bodies (Fig. 7). The peripherally distributed actin bodies give the impression that they are being extruded or budded off. Under the light microscope it is impossible to determine whether the peripherally located actin bodies are just interior or exterior to the sarcolemma (Figs. 6, *a* and *b*, 7, and 9 *a*).

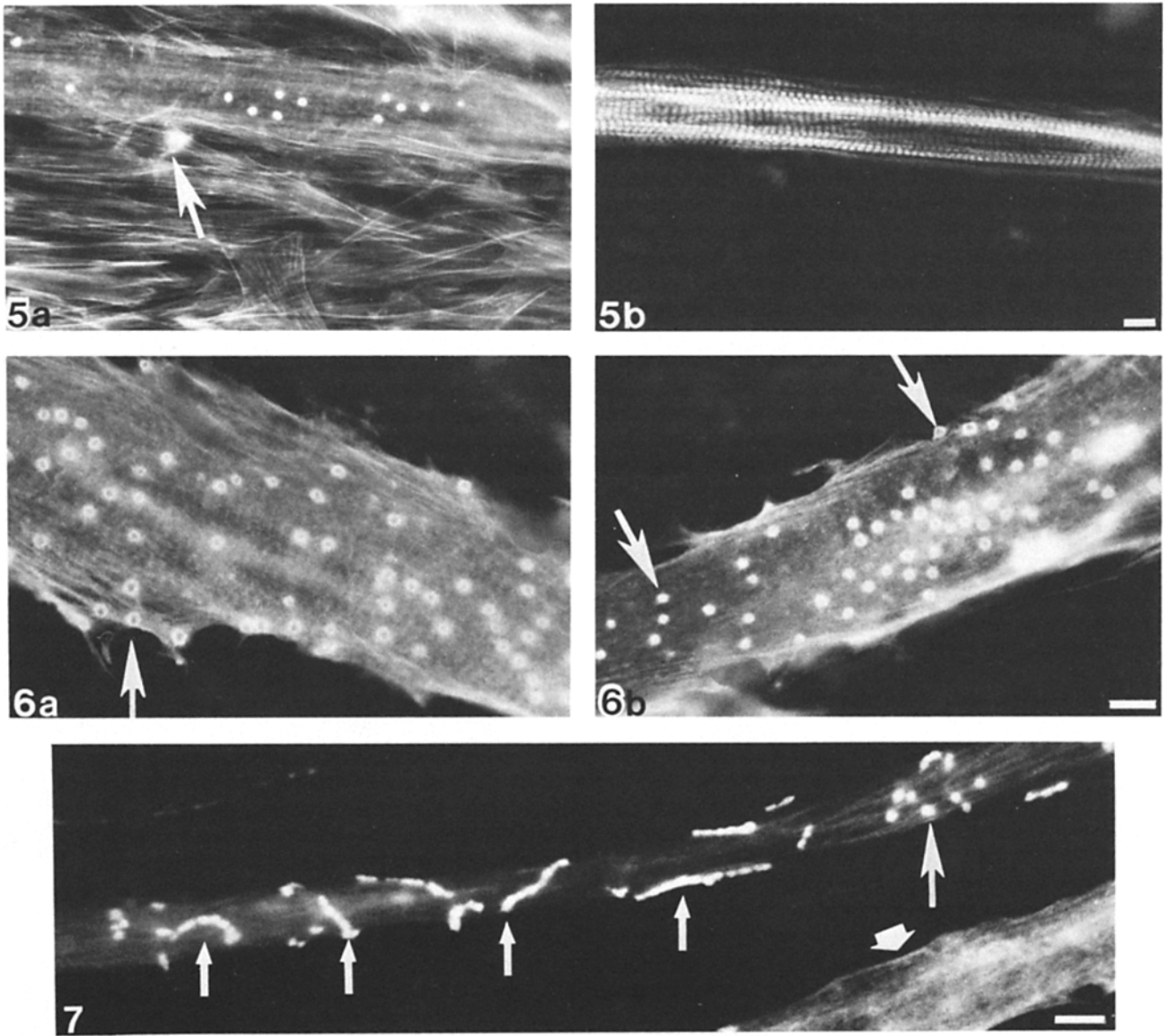
The number of actin bodies per myosac and the percent of myosacs per culture positive for bodies declines sharply after 10 h in TPA. They are present in only small numbers in 24-h myosacs, and all have disappeared in 48-h myosacs (Table I). Their decline is inversely associated with the appearance of muscle actin granules. These double stain with mAb B4 and rho-phalloidin and range from 0.2 to 2.0 μm. The larger granules cannot be distinguished from the actin disks. The

Table I. Ratio of Muscle Actin Bagels to Muscle Actin Disks

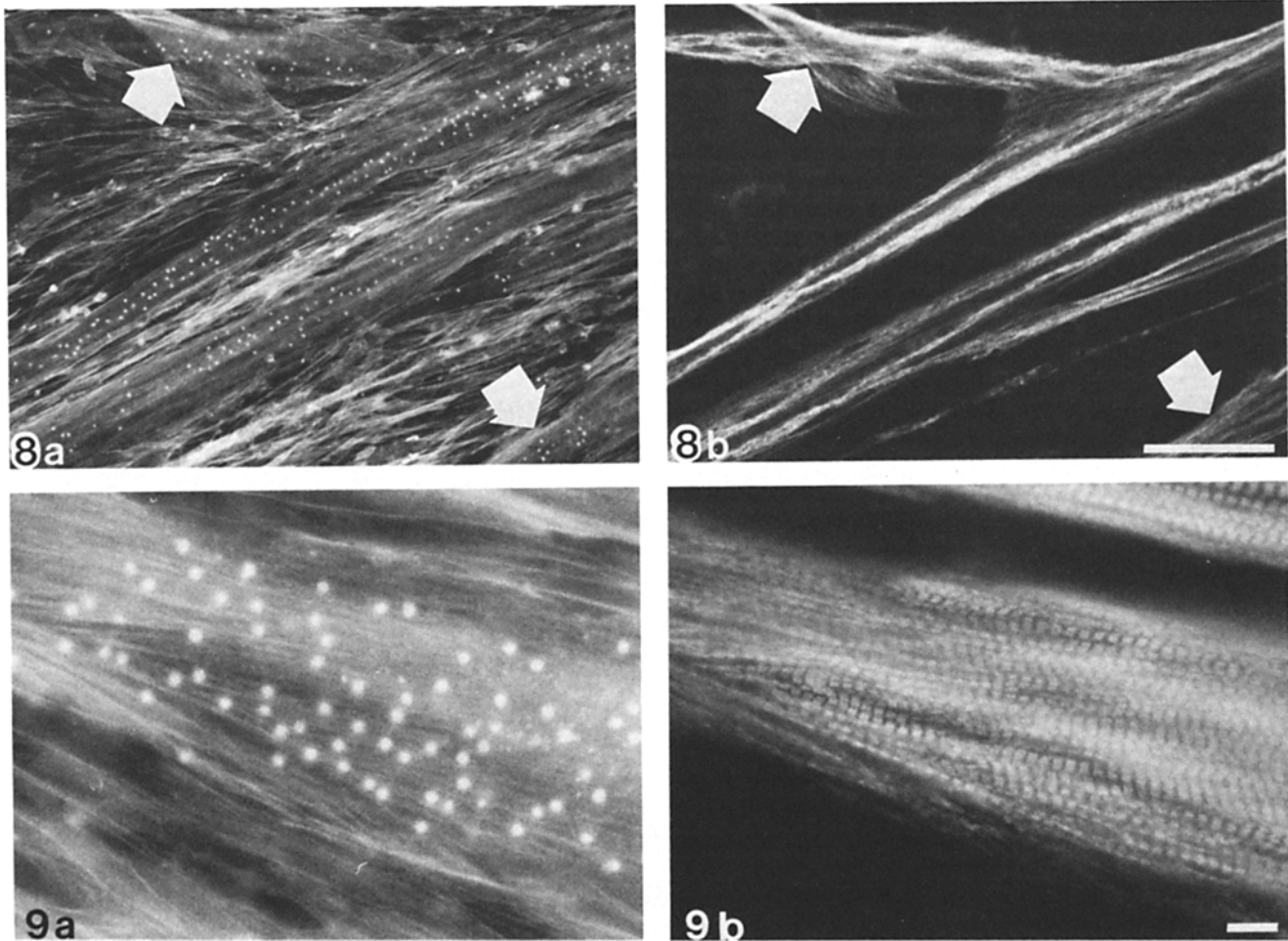
Time in TPA	Actin bagels	Actin disks	All myosacs with actin bagels and/or actin disks
<i>h</i>	%	%	%
5	70	30	80
10	15	85	73
24	5	95	5
48	Rare	Rare	<0.01
72	—	—	—

The precise time of appearance and disappearance of the muscle actin bodies varies from one series of cultures to another. Even in the same culture the emergence of actin bodies and retraction of myotubes into myosacs occurs earlier in relatively immature myotubes. The above counts are based on one typical series initiated with early day 4 cultures. 500–600 actin bodies were counted at random for each time point to determine the percentage of bagel-like to circular bodies. The figures for myosacs with vs. myosacs without bodies is based on the first 200 myosacs encountered. The same trend is observed in late day 4 cultures, but there is a delay of several hours in the appearance of the actin bodies as well as in their disappearance. The half-life of the individual actin bodies is unknown.

not shown). By permitting light from the fluorescein to enter the blue channel, MHC-positive material and the Hoechst 3342-stained nuclei can be visualized in the same micrograph (*c*). Lower right arrow points to a single mononucleated, fibroblastic cell that has crawled on top of the spread myosac; other arrows are for purposes of orientation. Note that several fibroblastic cells adjacent to the myosac also display prominent stress fibers and are MHC negative. Bar, 10 μm.



Figures 5-7. (Fig. 5, *a* and *b*) A 3-h myosac and surrounding mononucleated cells double-stained with rho-phalloidin (*a*) and anti-MHC (*b*, fluorescein channel). The rho-phalloidin 3.0- μ m bodies appear only in the myosac. Myofibrils still weakly bind the rho-phalloidin in a complex sarcomeric pattern but are out of focus. All of the flattened mononucleated cells display a rich complement of stress fibers. They do not form actin bagels, disks, granules, or even ribbons (cf. Figs. in reference 51). The arrow points to a ruffled membrane of a mononucleated cell. Ruffled membranes of the myotube's growth tip also stain brilliantly with rho-phalloidin (not shown). The pattern and distribution of the myofibrils visualized with anti-MHC is indistinguishable from those of untreated controls (*b*). The anti-MHC stains neither the actin bodies in the myosac nor the stress fibers in the adjacent fibroblastic cells. Bar, 10 μ m. (Fig. 6, *a* and *b*) Two 5-h myosacs and surrounding mononucleated cells, both stained with the mAb B4 to muscle actin. One (*a*) is visualized with a fluorescein-labeled, the other with a rhodamine-labeled secondary IgG (*b*). Most of the muscle actin bodies in both myosacs are still in the bagel-like configuration. The lower arrow in *b* points to a small cluster in the actin disk configuration. Alpha-actin I bands of contracted myofibrils are shown in *a*, whereas the myofibrils in *b* no longer bind the mAb B4. Compare the jagged borders of myosacs in TPA for 5 h with the smooth profiles of the control myotubes in Fig. 1 *a*. The mononucleated cells surrounding these myosacs do not stain with the muscle antiactin. The lower arrow in *a* points to several peripherally distributed actin bagels that appear to be located just beneath the sarcolemma. However, the location of the actin bagel with respect to the sarcolemma indicated by the upper arrow in *b* is less clear. The actin bagels and actin disks are oriented parallel to the undersurface of the myosacs. Reasons for describing these actin bodies as bagels or disks will be described elsewhere. Bar, 10 μ m. (Fig. 7) Portions of a mature (*large lower arrow*) and immature (*small arrows*) 5-h myosac stained with mAb B4 (rhodamine channel). Strings of contiguous actin bodies festoon the immature myosac. With this type of microscopy it is impossible to determine whether the actin bodies are inside or outside of the sarcolemma. Upper large arrow points to a more typical cluster of seven actin disks. Though not as numerous as in myosacs, contiguous strings of actin bagels or disks are also found in control cultures (see Discussion). Bar, 10 μ m.



Figures 8–9. (Fig. 8, *a* and *b*) Low power overview of a 10-h TPA-treated culture double-stained with rho-phalloidin (*a*) and anti-MHC (*b*, fluorescein channel). Rho-phalloidin no longer stains the thin filaments of the myofibrils in these myosacs (*a*). The A bands, however, appear normal when visualized with anti-MHC (*b*). The actin bodies are present only in myosacs; they are not induced in the adjacent, replicating presumptive myoblasts or fibroblasts. Not apparent at this low magnification are the occasional rho-phalloidin-positive, mAb B4-negative, ruffled membranes and blebs associated with the mononucleated cells. Arrows are for orientation. Bar, 100 μ m. (Fig. 9, *a* and *b*) A double-stained 15-h myosac that illustrates how differently the same myofibrils can appear, depending upon whether they are visualized so as to reveal alpha-actin in thin filaments or MHC in thick filaments. *a* illustrates the structures that stain with rho-phalloidin, *b* the structures that stain with anti-MHC. In addition to staining actin bodies, rho-phalloidin stains nonstriated fibers that, over the next 20 h, evolve directly or indirectly into typical long stress fibers as the myosac flattens. The A bands toward the right of the myosac in *b* appear normal, whereas the A bands toward the left are beginning to blur. Bar, 10 μ m.

granules are MHC negative. Actin disks and granules, and even occasional actin bagels, can be present in the same 24–36-h myosac, but by 48 h even the granules have disappeared (Figs. 3 *b* and 4 *a*).

Cultures of cardiac myocytes, smooth muscle, chondroblasts, liver, and nerve exposed to TPA and then stained with either mAb B4 and/or rho-phalloidin do not display actin bagels, disks, or granules of the kind described in TPA-myosacs (data not shown). Rho-phalloidin-positive, but mAb B4-negative, irregularly shaped, extracellular blebs, 4–7 μ m in diameter, are common in control and TPA-treated myogenic cultures as well as in cultures of nonmuscle cells.

Disassembly of Myofibrils and the Subsequent Elimination of Alpha-actin and MHC

Concurrent with the emergence of muscle actin bodies there is a sequential dismantling of the myofibrils. Changes in the

visualization of the muscle actin in I bands occur hours before such changes occur in the MHC in A bands. In approximately half the 5-h myosacs mAb B4 and rho-phalloidin stain both the I bands and the actin bodies (Figs. 5 *a* and 6 *a*). In the remaining 5-h myosacs that contain actin bodies, myofibrils cannot be visualized after being stained with the anti-muscle actin or rho-phalloidin (Figs. 6 *b* and 8 *a*). Less than 10% of the 10-h myosacs still reveal sarcomeric structures when stained with either anti-muscle actin or rho-phalloidin, whereas actin bodies are conspicuous in roughly 70% (Figs. 7, 8 *a*, and 9 *a*).

Double staining with combinations of anti-muscle actin or rho-phalloidin plus antibodies to MHC demonstrates that although sarcomeric structures associated with the thin filaments no longer stain in most 10-h myosacs, those associated with thick filaments appear to be normal (Figs. 5, *a* and *b*; 8, *a* and *b*; and 9, *a* and *b*). This contrast between the appearance of morphological integrity of myofibrils stained with

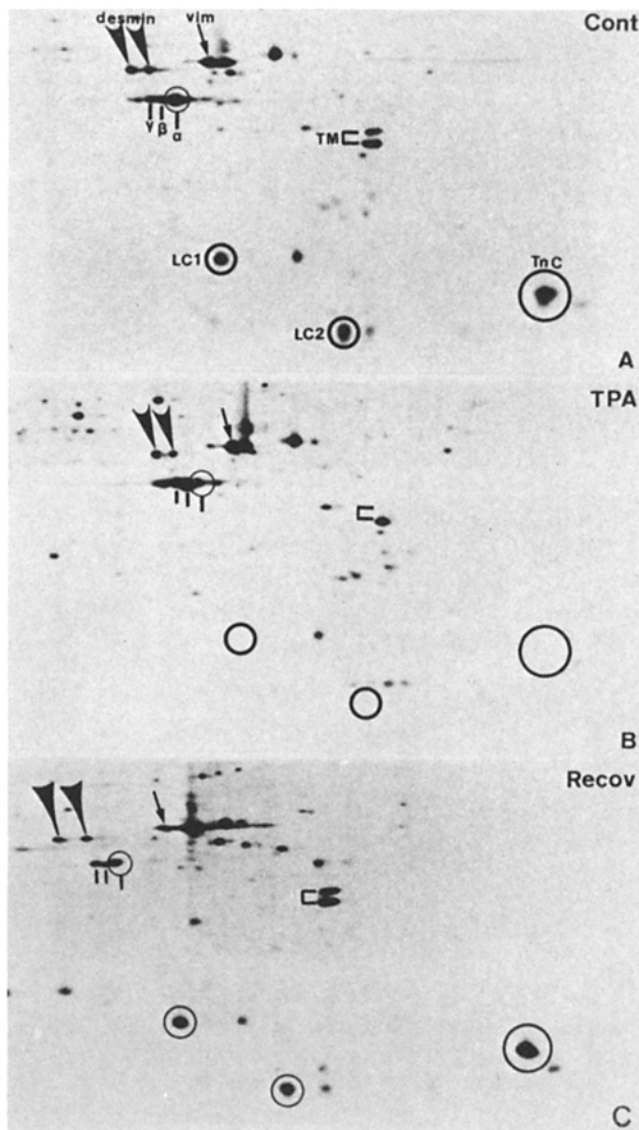


Figure 10. Fluorographs of two-dimensional polyacrylamide gels showing the proteins synthesized during a 1-h pulse of [³⁵S]methionine. (A) Day 4 control myogenic culture; (B) day 7 culture exposed to TPA from days 4–7; (C) culture treated as in B, but allowed to recover in normal medium for 2 d. *Vim*, vimentin; $\alpha/\beta/\gamma$, actin isoforms; *TM*, muscle-specific tropomyosins; *LC1* and *LC2*, myofibrillar myosin fast light chains 1 and 2; *TnC*, troponin C. The arrows and circles in B and C correspond to proteins labeled in A. Note the loss of synthesis of myofibrillar proteins in B and the resumption of their synthesis in C. Gels contain equal numbers of counts per minute. Basic proteins are to the left.

mAb B4 vs. anti-MHC is most evident in 10-h myosacs. In over 90% of 10-h myosacs neither rho-phalloidin nor anti-muscle actin reveal myofibrillar structures, whereas staining with anti-MHC appears normal. Myofibrillar breakdown, as visualized with anti-MHC, is first obvious in roughly half of the 15-h myosacs. This disassembly is heralded by gaps appearing between long stretches of still normally spaced, tandem A bands, as well as by blurring of their lateral borders (Figs. 8 b and 9 b). Rarely are distinct A bands still present in 24-h myosacs. At this stage, the considerable quantities of MHC amorphous material have collapsed into large, irregu-

lar clumps scattered throughout the body of the myosac. The pattern, size, and intensity of fluorescence in these MHC amorphous clumps varies from myosac to myosac (Fig. 4 b). They become less conspicuous in 48-h myosacs, and are completely cleared in roughly 70% of the 72-h myosacs. At no stage during the collapse of A bands into MHC aggregates do the latter costain with anti-muscle actin or rho-phalloidin (Fig. 4 a). Failure of the MHC-positive patches to costain with mAb B4 is not due to some staining artifact. These MHC aggregates costain with antibodies to C-protein, myomesin, and titin (our unpublished observations).

Emergence of Newly Assembled Stress Fibers in Myosacs

Rho-phalloidin-positive stress fibers are present in most flattened mononucleated cells in both control and TPA-treated cultures (Figs. 4 a, 5 a, 8 a, and 9 a). Many of the mononucleated cells that lack stress fibers tend to be rounded, less spread cells.

As mentioned earlier, the conspicuous stress fibers present in all normal day 2 and 3 myotubes largely disappear by day 4 (Fig. 1 b). In untreated day 4 and older myotubes, stress fibers are confined to the myotube's growth tips and to flattened, lateral pseudopodial extensions. In contrast, stress fibers begin to be assembled in 24-h and older myosacs (Fig. 4 a). The time of their reemergence varies among myosacs within a given culture and even within regions of the same myosac. When and where stress fibers assemble correlates more with degree of flattening than time in TPA. The central region of most early myosacs is rounded relative to the periphery. Where the nuclei cluster, the thickness varies from 3.0–6.0 μm ; at the periphery from 0.5–0.8 μm . If a region of a myosac has flattened, that region is apt to display stress fibers. This correlation between stress fibers in thin regions and their absence in thicker regions can be diagrammatically expressed within a single myosac. In most 24–72-h myosacs, stress fibers are restricted to the spread centrifugal regions and are absent over the central mass of the myosac; 24–72-h myosacs that have not spread lack stress fibers. In the more isodiametric, spread myosacs, the stress fibers can assume a radial or circumferential pattern.

Over 60% of the 36-h myosacs display various numbers of stress fibers. We determined whether protein synthesis is required for the reemergence of stress fibers that accompanies the flattening of myosacs. Cycloheximide was added to cultures that had been treated with TPA for 20 h. After an additional 10 h in both drugs, the myosacs were stained with the mAb B4 against muscle actin and/or rho-phalloidin. Many myosacs were rounded and in the process of detaching from the collagen substrate. Of the many that remained flattened, most displayed an arrangement of stress fibers indistinguishable from that in standard 30–36-h myosacs. These were mAb B4 negative, but stained in a punctate fashion with anti-CB (1, 2).

TPA Differentially, but Reversibly, Depresses the Synthesis of Myofibrillar but Not Cytoskeletal Proteins

Replicas of the myogenic cultures used for the cytoimmunofluorescence studies were pulsed with [³⁵S]methionine and the proteins they synthesized separated on two-dimensional gels. Four series were analyzed: (a) day 4 control cultures (e.g., Figs. 1 and 2); (b) day 7 cultures that had been treated

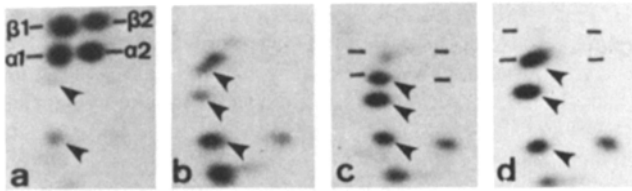


Figure 11. Comparison of tropomyosin regions, as separated on two-dimensional gels. 10-d control culture (a) synthesis. Control fibroblast culture (b) synthesis. TPA-treated culture (c) synthesis. TPA-treated culture (d) accumulation. Dashes in all figures correspond to muscle-specific tropomyosins labeled in a. $\alpha 1$ and $\beta 1$, nonphosphorylated forms of muscle-specific alpha- and beta-tropomyosins. $\alpha 2$ and $\beta 2$, phosphorylated forms of muscle-specific alpha- and beta-tropomyosins. Arrowheads indicate nonmuscle tropomyosins. Note that the nonmuscle tropomyosin of highest molecular weight comigrates with alpha 1 (see text). Acidic isoelectric points are to the right.

with TPA for the previous 72 hours, referred to as 72-h myosacs in this report (Fig. 4); (c) day 10 cultures that had been treated for 72 h with TPA, as in b, but then allowed to recover in normal medium for an additional 3 d (see Figs. in References 11, 12, 14, 58); (d) day 10 control cultures.

Day 4 control cultures synthesized alpha-actin, myofibrillar myosin fast light chains 1 and 2, and troponin C. They also synthesized desmin, vimentin, alpha- and beta-tubulins, and the nonmyofibrillar beta- and gamma-actins (Fig. 10 A). Day 10 control cultures synthesized the same ensemble of proteins (Fig. 10 C).

As shown in Fig. 10 B, day 4 cultures exposed to TPA for 3 d ceased synthesizing and accumulating these myofibrillar proteins. Fig. 11 shows the tropomyosin regions of gels prepared from such cultures labeled for either 1 h (lane c) or 48 h (lane d). The tropomyosins synthesized and accumulated by TPA-treated cultures were of the fibroblast type (45). Comigration of cold muscle tropomyosin standards with radioactive fibroblast or TPA-treated samples showed that the nonphosphorylated form of alpha-tropomyosin ($\alpha 1$) colocalizes with the heaviest nonmuscle tropomyosin. The immune blot in Fig. 13 confirms that the myofibrillar tropomyosin isoforms are either absent or in very low quantities in TPA-treated muscle cultures.

TPA reversibly blocked the synthesis and accumulation of troponin C (Fig. 10, B and C). Protein immunoblots revealed very low levels of muscle actin in 72-h TPA-myosacs (Fig. 12). In contrast, the synthesis of the nonmyofibrillar beta- and gamma-actins was not blocked (Fig. 10 B).

Fig. 14 indicates that TPA-treated cultures (lane b) contain greatly reduced amounts of Triton-insoluble MHC. Lane d in Fig. 14, which contains ~ 15 times more protein than lane b, displays an MHC band. Clearly, little myofibrillar MHC is synthesized in 72-h myosacs. The presence of modest amounts of antigenically active MHC still present in 72-h myosacs is most likely due to the amorphous clumps of MHC-positive material.

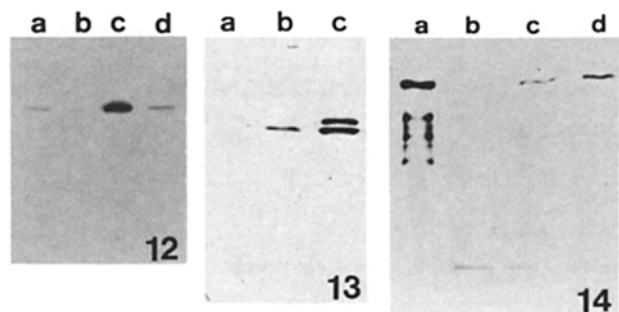
Although TPA greatly reduced the synthesis and accumulation of myofibrillar proteins, it did not markedly increase or decrease the synthesis of desmin, vimentin, tubulin, or the synthesis of beta- or gamma-actins (Fig. 10 B). Such independent regulation of myofibrillar and desmin protein synthesis has also been observed in normal muscle cultures (2,

16, 24, 28), and muscle cultures exposed to the carcinogen ethyl-methylsulfonate (EMS) or to tetrodotoxin (1, 9). The normal appearance of desmin, vimentin intermediate filaments, and microtubules in 72-h myosacs as well as the normal accumulation of SR and T-system components, was emphasized in earlier EM and cytoimmunofluorescence studies (1, 2, 11, 28, 58). These findings demonstrate that irrespective of what other processes it might affect, TPA selectively depresses the synthesis of many (all?) myofibrillar proteins.

Discussion

The assembly of beta- and gamma-actins into stress fibers is likely to be subject to controls different from those ordering the assembly of alpha-actin into myofibrillar thin filaments. It is also clear that the conditions in TPA-myosacs that preclude the assembly of alpha-actin filaments for myofibrils permit the assembly of beta- and gamma-actin filaments for stress fibers. The demands on the relatively stable, 1.0- μm long, uniformly polarized alpha-actin filaments in myofibrils differ from those made on stress fibers or microfilaments. It is an open question, then, as to the applicability of data derived from the behavior of beta- and gamma-actins in non-muscle cells (34, 36, 37, 52, 56) to the structuring of thin filaments for myofibrils. Even if identical cutting and capping proteins function in the assembly of muscle actin thin filaments, questions remain as to how such regulatory proteins are positioned with respect to Z bands, myosin cross-bridges, etc. TPA-recovering myosacs should be useful for analyzing how alpha-actin is packaged into polarized thin filaments with their associated regulatory proteins.

Thus far, the literature has stressed the similarities rather than the differences in actin isoforms (20, 23, 37, 42, 59, 60). Accordingly, the rapid susceptibility to TPA of the alpha-actin in myofibrillar thin filaments versus the resistance of



Figures 12-14. (Fig. 12) Comparison of amount of Triton-insoluble (lanes a and c) and Triton-soluble (lanes b and d) alpha-actin which is present in TPA-treated (lanes a and b) vs. recovered cultures (lanes c and d). Lanes a and b contained 10 times the amount of radioactivity as used for lanes c and d. (Fig. 13) Muscle tropomyosins are absent in whole cell samples of TPA-treated muscle (lane a) but are present in cultures recovered in normal medium for 1 d (lane b) or 3 d (lane c). All lanes contain equal counts per minute. (Fig. 14) Amount of Triton-insoluble MHC present in TPA-treated cultures (lanes b and d) and cultures recovered in normal medium for 2 d (lane c). Lane a is a myosin standard. Lanes b and c contain equal amounts of radioactivity, whereas lane d contains 15 times more counts per minute than lane b. The relative migrations of proteins in the different immunoblots cannot be compared because each gel contains a different percentage of acrylamide.

the beta- and gamma-actin in stress fiber thin filaments is of interest. Virtually equivalent as molecules both *in vivo* and *in vitro*, actin isoforms can behave differently in cells depending on what molecules they are associated with, what structures they become part of, or even in which type of cells these structures are assembled. For example, (a) although cytochalasin B promptly aborizes fibroblasts and presumptive myoblasts, severing their stress fibers, it does not have a similar effect on myofibers in skeletal or cardiac muscle cells (10, 27, 31, 33); (b) stress fibers in chondroblasts are short, thin, and frequently overlap, whereas in hepatocytes they are broad, long, and converge on junctional complexes (Duran, S., Z. Lin, and H. Holtzer, unpublished observations); and (c) in maturing cardiac myocytes and postmitotic skeletal cells, stress fibers are prominent, but transient. Early in myofibrillogenesis nonmyofibrillar and myofibrillar isoforms of actin, MHC, alpha-actinin, and tropomyosins polymerize and coexist in the same narrow subsarcolemmal domains, possibly forming heteropolymers (1, 15, 28, 39). With further maturation, however, the conditions that favor accumulation, stabilization, and selective interactions of myofibrillar isoforms favor the disassembly of the nonmyofibrillar isoforms of stress fibers (1, 15).

That the phenotypic effects of TPA on different cell types (eg. 29, 30, 40, 49) is a function of the ongoing differentiation program of the responding cell is documented by the different responses of (a) stress fibers in immortalized vs. primary cells, and (b) myofibrils in cardiac myocytes vs. myotubes. TPA disassembles the stress fibers in immortalized BSC-1 and PTK 1 cells within 60 min (43, 51). In contrast, in our primary cultures there was no gross effect on stress fibers of fibroblasts, presumptive myoblasts, or older myosacs, even after 72 h in TPA. These differences are not due to culture media or concentration of TPA. Primary fibroblasts, presumptive myoblasts, or TPA-myosacs did not disassemble their stress fibers when cultured in the same medium, or even at three times the concentration of TPA used for BSC-1 and PTK 1 cells (data not shown). Equally germane is the finding that TPA did not block the contraction of beating cardiac myocytes, nor compromise the integrity of their myofibrils. Stress fibers in immortalized cells, or as transitory structures in early normal myotubes and cardiac myocytes, probably accommodate to extracellular conditions and participate in intracellular events in ways different than those of stress fibers in presumptive myoblasts and fibroblasts. These phenotypic differences could be due to reduced numbers or down-regulation of TPA receptors, or even the absence of a class of proteases activated by a second messenger.

It will be interesting to follow the origins and fate of the actin bodies with time. Their rapid formation, sizeable number, uniform size and shape, and the fact that they do not fuse with one another is compatible with the notion that they derive from I-Z-I complexes of the disassembling myofibrils. Work to be reported elsewhere (Lin, Z., D. Fischman, W. Franke, and H. Holtzer, manuscript in preparation) demonstrates that the actin bagels are positive for alpha-actinin and girdled by a discontinuous layer of vinculin. It is also likely that, at least initially, they are not membrane bound. Staining with rho-phalloidin suggests that the alpha-actin in the bodies is still polymerized. Whether the loss of the actin bodies and actin granules involves active extrusion or involvement of the lysosomal system is being investigated. The formation and clearance of the MHC amorphous patches

is not associated spatially or temporally with the alpha-actin bodies. The cytoimmunofluorescence and immunoblot data demonstrate that the MHC in the patches is slowly degraded, probably to amino acids. Together, these findings suggest surprisingly distinct pathways for the degradation of these major classes of myofibrillar proteins.

The spatial and temporal segregation of breakdown of alpha-actin and myofibrillar MHC in TPA-myosacs may be relevant to normal turnover of myofibrillar proteins (19, 21, 22, 65). TPA activation of protein kinase C may mobilize the Ca^{2+} -dependent neutral protease in Z bands, thus releasing alpha-actinin (50). This could result in the loss of a capping protein and lead to the packaging of small cohorts of thin filaments into the equivalent of actin bagels. Hours later those thick filaments that had formed cross-bridges with the removed thin filaments would collapse into the equivalent of MHC amorphous clumps. In brief, TPA need not induce any novel pathway in the responding myotubes. It might simply synchronize and enhance myofibrillar turnover, a process that occurs at a slow rate in normal myotubes. Preliminary observations support this view. Alpha-actin bodies are not uncommon in normal myotubes. They are particularly evident in early and slowly maturing, as well as spontaneously degenerating, control myotubes. They differ from those in TPA-myosacs in that they are (a) larger and less regular in geometry, and (b) present in fewer myotubes per culture.

In experiments to be reported elsewhere, we found that in TPA-myosacs, (a) the temporal and spatial clearance of titin followed that of MHC, myomesin, and C-protein, rather than alpha-actin and alpha-actinin; and (b) the number of transcripts for MHC and alpha-actin was drastically reduced after only 24 h in TPA. These mRNAs reappeared after 24 h of recovery in normal medium. To what degree resorption of myofibrillar structures induced by TPA reflects active proteolysis, rather than normal turnover not balanced by normal synthesis, remains to be determined.

Currently, desmin is the only known muscle-specific isoform synthesized by both replicating and postmitotic myogenic cells (16, 24, 28). Little is known of the developmentally regulated events that alter the transcription of either single or coordinated subsets of myofibrillar genes (13, 17). The succession of myofibrillar isoforms associated with maturation is affected by neural influences, cell-matrix interactions, and hormones. This type of readily reversible physiological regulation differs, we believe, from the relatively irreversible, autonomous reprogramming that occurs between replicating presumptive myoblasts and their postmitotic progeny. The latter involves up-regulation of most (all?) myofibrillar isoforms as well as of membrane molecules affecting fusion and components of the sarcoplasmic reticular (SR) and transverse tubular system (T-system). It also involves the down-regulation of DNA synthesis and loss of centrosomes and microtubule-organizing centers (reference 54; Lin, Z., and H. Holtzer, unpublished observations). Normally, this transition is an all-or-none event. In immortalized and partially transformed myogenic cells such as L6, L8, and L6E9, this sharp distinction between replicating precursor and postmitotic daughters is lost (contrast data and concepts in references 44, 46, 61, 63 with those in references 13, 25, 32). Recently, it was shown that the normally integrated differentiation program of postmitotic myogenic cells could be fractionated into two relatively independent parts. Postmitotic myogenic cells reared in the mutagen EMS

continue to synthesize desmin, fuse into multinucleated myosacs, assemble prominent stress fibers and SR and T-system elements, and lose their microtubule-organizing centers. EMS-myosacs, however, do not synthesize myofibrillar isoforms (1, 2, 28). The relatively selective, but reversible, effects of TPA and EMS on myofibrillogenesis are similar to the reversible blocking effects of ts-RSV on postmitotic myotubes (25, 29, 30, 55). That three different perturbing agents, TPA, EMS, and pp60 v-src, selectively, but reversibly, block the synthesis of most (all?) myofibrillar proteins suggests a curious vulnerability of a normally coupled subset of myofibrillar genes that constitute a sizeable fraction of the terminal myogenic program. It is important to distinguish between genetic mechanisms that (a) regulate the assembly of a cell's differentiation program at the time of its birth independently of exogenous molecules, and (b) modulate the expression of the inherited differentiation program in response to exogenous molecules like TPA, retinoic acid, DMSO, hormones, collagens, growth factors, etc.

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