Effects of taxol and Colcemid on myofibrillogenesis

(microtubules/Z-bands/thin filaments/thick filaments/intermediate filaments)

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ABSTRACT To determine the relationship between thin filaments, Z-bands, microtubules, intermediate filaments (IFs), T-tubules, and sarcoplasmic reticulum (SR) during myofibrillogenesis, myotubes were selectively depleted of their myofibrils with 12-tetradecanoylphorbol 13-acetate (TPA) and then were allowed to regenerate in (i) normal medium, (ii) taxol, and (iii) Colcemid. Myofibrils assembled in normal medium formed typical A-, I-, Z-, M-, and H-bands and associated IFs, T-tubules, and SR. Myofibrils assembled in taxol formed "A-bands" of aligned thick filaments interdigitating with long microtubules and "I-bands" consisting only of microtubules. These unprecedented sarcomeres lacked thin filaments, Z-bands, and associated IFs and SR. "Solitary A-bands," consisting exclusively of laterally aligned bipolar thick filaments 1.6 μM in length without either thin filaments or microtubules, were observed. Myofibrils assembled in Colcemid formed all myofibrillar components in the absence of microtubules but these did not achieve rigorous lateral alignment. Colcemid and taxol induced the formation of patchy Z-bands that invariably served as insertion sites for thin filaments, irrespective of the presence or absence of adjacent thick filaments. Z-bands may function as actin-organizing centers for each sarcomere.

Vertebrate striated myofibrils are strikingly invariant. Relaxed sarcomeres display A-, I-, Z-, M-, and H-bands. Constancy of these repeat units is the result of the assembly and alignment of thick and thin filaments into interdigitating hexagonal arrays (1–3).

Analysis of myofibrillogenesis by using labeled antibodies against muscle-specific isoforms has suggested that, after a particular quantal cell cycle, muscle-specific genes for myosin heavy and light chains, tropomyosin, and desmin are coordinately activated in *postmitotic, mononucleated myoblasts* and their translation products are rapidly assembled into thick and thin filaments (4–11). Nascent myofibrils are associated with microfilaments subtending the plasmalemma and the lengths of their A- and I-bands are identical to those in mature muscle. New sarcomeres are added at the ends of myofibrils proximal to the growth tips of the cell. Growth in width occurs by the peripheral apposition of newly polymerized thick and thin filaments. This growth involves myofibril displacement from the cell cortex into the interior, whereas the same myofibril maintains its distal association with microfilaments in the growth tips (4, 6, 7, 12–17). Electron microscopic studies also emphasize that nascent myofibrils arise by the interdigitation of polarized thick and thin filaments. Microtubules and 10-nm filaments (intermediate filaments, IFs) surround, and the sarcoplasmic reticulum (SR) and T-tubules engage, these early myofibrils (4, 8, 17–21).

To determine the possible scaffolding roles of microtubules, Z-bands, and IFs in the assembly of sarcomeres, as well as the capacity of myosin and α -actin monomers to self-assemble, ex-

periments were performed using three drugs. The first drug, the cocarcinogen 12-tetradecanoylphorbol 13-acetate (TPA), selectively degrades striated myofibrils in immature myotubes (22–24). Upon removal from TPA, these myofibril-depleted myotubes rapidly synthesize and coordinately assemble myofibrillar proteins into striated myofibrils. The second drug, taxol, stabilizes microtubules (25, 26). Taxol induces the formation of myofibrils that sometimes consist not of interdigitating thick and thin filaments but of interdigitating thick filaments and microtubules (4). The third drug, Colcemid, induces elongated postmitotic myoblasts and myotubes to form myosacs and disrupts the alignment of thick and thin filaments; it also induces patchy Z-bands (i.e., nemaline bodies) with inserted thin filaments and aggregates vimentin and desmin IFs into cables (9, 15, 17, 18, 23, 27).

Given these selective but reversible effects of TPA, taxol, and Colcemid on myofibrillogenesis, the following experimental design was used. Day 3 myotubes that had initiated the assembly of myofibrils were cultured for 2 additional days in TPA. These day 5 myotubes, largely depleted of all thick and thin filaments and Z-bands, were divided into three groups. The first group was allowed to recover in normal medium to confirm the fact that they would assemble normal, striated myofibrils (22, 23). The second group was allowed to recover in normal medium with taxol to reveal whether the assembly of thin filaments was dispensable for sarcomere formation. The third group was allowed to recover in normal medium with Colcemid to determine the role of patchy Z-bands, microtubules, and IFs in the assembly of sarcomeres.

MATERIALS AND METHODS

Primary myogenic cultures of chicken muscle were prepared as described (4, 27). Day 3 cultures were treated with TPA (final concentration, 0.01 μM) for 48 hr. The cultures were washed and reared for an additional 2–6 days in (i) normal medium, (ii) normal medium with taxol (final concentration, 10 μM), or (iii) normal medium with Colcemid (final concentration, 1 μM). Electron microscopy was performed as described by Toyama *et al.* (24). Actin was localized with heavy meromyosin S1 (HMM-S1) as described (28) by varying the technique introduced by Ishikawa *et al.* (19). HMM-S1 was a kind gift of A. Weber (University of Pennsylvania). Taxol was obtained from the Natural Products Branch, Division of Cancer Treatment, National Institutes of Health.

RESULTS

TPA-Depleted Myotubes Regenerating in Normal Medium. The selective degradation of virtually every myofibril in early

Abbreviations: IFs, intermediate filaments; SR, sarcoplasmic reticulum; TPA, 12-tetradecanoylphorbol 13-acetate; HMM-S1, heavy meromyosin S1.

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myotubes by TPA has been described (22, 23). Electron micrographs reveal only rare scattered clumps of thick and thin filaments. These depleted myotubes are filled by a tangled web of IFs in which SR, T-tubules, and mitochondria are randomly suspended. When returned to normal medium, every myotube rapidly assembles a normal complement of striated myofibrils, which contract spontaneously. Every sarcomere consists of interdigitating thick and thin filaments, assembled into A-, I-, Z-, M-, and H-bands which are indistinguishable from those in mature muscle (Fig. 1*a*). The SR and a modest number of microtubules and IFs that course parallel to, and between, the regenerated myofibrils are similar to those in untreated day 9 myotubes.

TPA-Depleted Myotubes Allowed to Regenerate in Taxol. Most sarcomeres are greatly deficient in Z-band material and lack thin filaments as well (Figs. 1*b* and 2*a* and *b*). What appear as normal A-bands prove to be aligned thick filaments interdigitating with long microtubules that are the only structural elements in the "I-bands" of these sarcomeres. In sarcomeres lacking Z-band material and thin filaments, there is an exclusion of SR, T-tubules, and IFs from the myofibrillar domain. Although present, the SR, T-tubules, and IFs appear to be

FIG. 1. Low-power electron micrographs of TPA-treated myotubes reared for 4 days in (a) normal medium ($\times 6,600$), (b) normal medium with taxol ($\times 6,600$), or (c) normal medium with Colcemid ($\times 7,400$). (a) Note the perfectly normal regenerated striated myofibrils. (b) A- and I-bands give the impression of reasonably normal sarcomeres. However, note the almost total absence of Z-band material. Two patchy Z-bands are indicated by arrows. (c) The major portion of these myosacs (15, 27) displays all the structures associated with myofibrils but these are greatly disorganized. In other regions of the same myotube, particularly subjacent to the sarcolemma, organized sarcomeres are present.

shunted into areas of the sarcoplasm which lack myofibrils.

The absence of thin filaments or G-actin (or both) in these thick filament-microtubule arrays was verified by tilting electron microscopy sections and by decorating with HMM-S1. Details of these experiments will be published elsewhere. Briefly, no thin filaments were detected when paired sections were viewed stereoscopically and no arrowhead complexes were observed among the interdigitating thick filament-microtubule arrays. However, sizeable skeins of decorated actin filaments were observed in their vicinity. Whether these decorated filaments consisted originally of α -, β -, or γ -actin monomers that were induced to polymerize by treatment with HMM-S1 (19) is currently unknown.

Some regions in these myotubes display relatively normal sarcomeres. Quantitation of those that exhibit normal Z-bands; patchy Z-bands, or no Z-bands is at best a crude approximation. Roughly 60% of all sarcomeres lack all Z-band material, 30–40% display small to sizeable patchy Z-bands, and 5–10% form normal Z-bands. The latter display a bilateral arrangement and normal density of polarized thin filaments; these interdigitate with flanking bipolar thick filaments to form hexagonally stacked thick and thin arrays. Of more interest theoretically are the scattered patchy Z-bands. Even the smallest of these serve as insertion sites for thin filaments. Where this occurs, the flanking A-bands reveal variable numbers of interdigitating thick filaments, microtubules, and thin filaments (Fig. 2*c*). Where patchy Z-bands and associated thin filaments are present, SR, T-tubules, and IFs are also found.

Pertinent to the relationship between self-assembly and myofibrillogenesis are the remarkable "solitary A-bands" (Fig. 2*d*). These highly ordered structures are randomly oriented and display no obvious relationship to any structure in the myotube.

TPA-Depleted Myotubes Allowed to Regenerate in Colcemid. In these myotubes, although all components associated with myofibrils regenerate, their relative ratios and spatial relationships are distorted (Figs. 1*c* and 3*a–c*). These myofibrils characteristically display longitudinally staggered thick filaments irregularly interrupted with variably sized patchy Z-bands with attached polarized thin filaments. Thin filaments attached to patchy Z-bands may or may not engage nearby poorly aligned thick filaments. In many regions with patchy Z-bands, there is an increased ratio of thin to thick filaments and these are interspersed with IFs. "Solitary patchy Z-bands" with only long inserted thin filaments are numerous. In many regions the ratio of interdigitating thick to thin filaments approaches normal: in such regions the morphology and size of the Z-bands approaches normal. Microtubules are not present in these myotubes.

The ratio of organized to disorganized myofibrils is higher in myotubes that regenerated in Colcemid for 6 days than in those that regenerated for 4 days (data not shown). It is important to determine why Colcemid's effect in interfering with myofibrillogenesis diminishes with time.

DISCUSSION

The relationships between sarcomere structures, self-assembly of myosin monomers into thick filaments of the appropriate length, and the possible role of Z-bands as actin-organizing centers have been the subject of many investigations (1–4, 7, 15, 16, 29–35).

Experiments with taxol demonstrate that, in the absence of both thin filaments and Z-bands, myosin monomers can self-assemble into thick filaments $\approx 1.6 \mu\text{m}$ in length and that these will align laterally. The presence of M- and H-bands in the solitary A-bands and in interdigitating arrays of thick filaments and

FIG. 2. Electron micrographs of myofibrils assembled in TPA-treated myotubes that recovered in taxol for 4 days. (a) The individual thick filaments are $\approx 1.6 \mu\text{M}$ in length and the M- and pseudo-H-bands have the same dimensions as the corresponding bands in normal A-bands. The same microtubules that constitute the only visible structures of the "I-bands" (arrowheads) interdigitate with the flanking thick filaments, which form a remarkable facsimile of normal A-bands. Single microtubules extend through several consecutive sarcomeres. The arrow points to a cluster of parallel microtubules of indefinite length. Elsewhere in this myotube the lateral alignment of the thick filaments may be less precise. No structures that might be analogous to microtubule-organizing centers were observed with the microtubules of these striated pseudo-myofibrils. ($\times 51,000$.) (b) A cross section illustrating the absence of all thin filaments in interdigitating arrays consisting only of thick filaments and microtubules. ($\times 116,000$.) (c) A cross section through a sarcomere displaying interdigitation of thick and thin filaments and microtubules. The number of thin filaments in such arrays varies directly with the size of the Z-band material. The actual numbers of thin filaments/thick filaments/microtubules in four different cross sections are 2:17:22, 0:29:11, 19:22:14, and 0:27:9. ($\times 115,000$.) (d) A longitudinal section through a solitary A-band. The precise lateral alignment of thick filaments reveals the M- and H-bands. Cross sections and stereo pairs of such structures confirm the total absence of interdigitating thin filaments or microtubules. In cross sections the thick filaments in these structures tend to line up in parallel rows. ($\times 58,000$.)

microtubules suggests that intracellular polymerization is initiated by tail-to-tail interaction, followed by tail-to-head addition of monomers as it is *in vitro*.

It is also clear that thick filaments will align with microtubules, structures with which they are not known to interact intracellularly (4). Evidence for the *in vitro* interaction of thick filaments with microtubules is provided by the work of Hayashi and co-workers (36, 37). They found that the affinity of tubulin dimers for synthetic myosin filaments equaled that of actin but that the decoration of such filaments differed from that of F-actin-myosin binding in being unaffected by Ca^{2+} or MgATP. Preliminary experiments suggest that even *in vitro*, taxol enhances the interaction between myosin filaments and poly-

merizing tubulin dimers (unpublished data).

Whether the alignment of thick filaments in solitary A-bands is due to M-band or C-protein is unknown. Although we favor the notion that solitary A-bands self-assemble from myosin and M-band protein monomers (4), we have not excluded the possibility that they arise secondarily—either by displacement from associated microtubules by a form of sliding movement or by depolymerization or degradation of the interdigitating microtubules even in taxol. More challenging with respect to macromolecular interactions is the way in which aligned, bipolar thick filaments that interdigitate with long microtubules of presumably unidirectional polarity maintain a distance one from another roughly equivalent to a normal I-band. What type of

FIG. 3. Electron micrographs of myofibrils assembled in TPA-treated myotubes or myosacs that recovered in Colcemid for 4 days. (a and b) The thin filaments issuing from the numerous patchy Z-bands form loose bundles that interdigitate with adjacent thick filaments to varying degrees. Often thin filaments and IFs intermingle. The thick filaments are not only poorly aligned laterally but are staggered longitudinally. Often single or small clusters of poorly aligned thick filaments, with or without neighboring thin filaments, angle across adjacent thick filaments. The total area occupied by the numerous patchy Z-bands in these disorganized myofibrils exceeds that occupied by Z-bands in normal myofibrils. Contributing to this spatial disorder is the random distribution of SR and T-tubules. This disarray of myofibrillar elements is consistent with the failure to observe sarcomeres in Fig. 1c. Note in b how some myofibrils have been sectioned longitudinally, whereas others appear almost as cross sections (a, $\times 9,600$; b, $\times 15,000$.) (c) A longitudinal and a cross section through two patchy Z-bands. Arrows point to associated thin filaments. Note the total absence of thick filaments. ($\times 64,000$.)

periodic signal might exist on the surface of microtubules? Are microtubule-associated proteins involved?

Little is known about the conditions that regulate polymerization-depolymerization equilibria of thick and thin filaments and how these affect the turnover of the different myofibrillar proteins. For example, will the failure of the monomers of α -actin, tropomyosin, and the troponins to become incorporated into thin filaments feed back on their respective rates of synthesis or degradation (or both)? Similarly, how stable are the thick filaments that interdigitate with microtubules compared with those that interdigitate with thin filaments? A related issue is the observation of more organized myofibrils in myotubes that have regenerated in Colcemid for 6 days compared with the

disorganized myofibrils present in myotubes that have regenerated in Colcemid for only 4 days. Is the uptake of Colcemid lower, or its possible intracellular degradation greater, after 6 days in contrast to briefer exposures? Are the disorganized myofibrils in the center of the myotube or myosac degraded preferentially, allowing the organized myofibrils assembled at the periphery to enlarge, possibly by serving as a template for the apposition of newly polymerized thick and thin filaments (7, 13–16)?

In addition to Colcemid, cytochalasin B and trypsin also induce patchy Z-bands (ref. 15; unpublished data). Generally, they have been interpreted as being partially digested fragments of injured myofibrils. However, our data are consistent

with the view that the molecules constituting these dense structures self-assemble, possibly only in the presence of actin monomers, and then they function as actin-organizing centers for each sarcomere. Evidence for this is the correlation between (i) the absence of Z-band material and the absence of thin filaments in taxol-treated cells and (ii) the presence of patchy Z-bands and associated thin filaments in both taxol- and Colcemid-treated cells. Comparable dense bodies associated with the β - and γ -actins in microfilaments have been described in chondroblasts and fibroblasts (15) as well as with actin filaments in smooth muscle (38). It will be of interest to determine whether taxol or Colcemid alters the relationship of such dense bodies and their associated filaments in these cell types.

The interaction of microtubules and thick filaments induced by taxol may be an intracellular artifact. Alternatively, if taxol stabilizes microtubules as they normally polymerize, then the drug is perpetuating a visible record of where microtubules formed in the past history of the cell. If this is true, then not only are microtubules constantly being assembled and dismantled in mononucleated postmitotic myoblasts (4) and myotubes but this cyclical activity is also influencing the polymerization or spatial deployment (or both) of the myofibrillar proteins.

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