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Inhibitors Arrest Myofibrillogenesis in Skeletal Muscle Cells at Early Stages of Assembly

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A three-step model for myofibrillogenesis has been proposed for the formation of myofibrils [Rhee et al., 1994: *Cell Motil. Cytoskeleton* 28:1–24; Sanger et al., 2002: *Adv. Exp. Med.* 481:89–105]: premyofibril to nascent myofibril to mature myofibril. We have found two chemically related inhibitors that will arrest development at both the first and second step. Cultured quail embryonic skeletal myoblasts were treated with ethyl methane sulfonate (EMS) or 2-aminoethyl-methanesulfonate ($MTSEA^+$). When the myoblasts fused in the presence of either of these compounds, myosheets rather than myotubes formed. Treated cells were fixed and immunostained against multiple proteins commonly found in muscle cells. Protein expression and localization throughout the myosheet were similar to that of developing myotube tips. Cells treated with high concentrations of EMS (10 mM) stained for non-muscle myosin II, sarcomeric alpha-actinin, and tropomyosin. No zeugmatin (Z-band region of titin) or muscle myosin II antibody staining was detected in fibers in this treatment group. These fibers are comparable to premyofibrils in control myotubes. At lower concentrations of EMS (7.5 to 5 mM), fibers that formed stained for muscle myosin II and titin as well as for non-muscle myosin IIB, sarcomeric alpha-actinin, and tropomyosin. Muscle myosin II was in an unbanded pattern. These fibers are comparable to nascent myofibrils observed during normal myofibrillogenesis. Similar effects to those obtained by treating cells with EMS were obtained when we treated cultured cells with $MTSEA^+$ (5 mM) and stained them with sarcomeric alpha-actinin. $MTSEA^+$ is chemically related to EMS, and is a well-known inhibitor of ryanodine receptors in skeletal muscle cells. Some abnormalities such as nemaline-like rods and other protein aggregates also appear within the myosheet during EMS and $MTSEA^+$ treatment. Removal of these two inhibitors of myofibrillogenesis allows the premyofibrils and nascent myofibrils to form mature myofibrils. Cell Motil. Cytoskeleton 59:1–16, 2004.

Key words: premyofibrils; nascent myofibrils; mature myofibrils; sarcomere; Z-bands; Z-bodies; alpha-actinin; non-muscle myosin II; muscle myosin II; titin; tropomyosin; troponin T; zeugmatin; sarcomere; ryanodine receptor; inhibitors

INTRODUCTION

Myofibrillogenesis is the process by which proteins in developing muscle cells form sarcomeres. Cultured skeletal myoblasts fuse and grow to form long, multinucleated cells. Developing myotubes have mature myofibrils in their centers and towards both leading edges until the very tips, where proteins form nascent myofibrils and premyofibrils [Rhee et al, 1994; Sanger et al., 2002]. A three-step model for myofibrillogenesis has been proposed for the formation of myofibrils [Rhee et al., 1994; Sanger et al., 2002]: premyofibril to nascent myofibril to mature myofibril. Premyofibrils are composed of minisarcomeric arrangements and lay the initial stages for building sarcomeres [Rhee et al., 1994]. The boundaries of the minisarcomere are composed of Z-bodies contain-

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ing sarcomeric alpha-actinin. Mini-A-bands composed of non-muscle myosin II filaments are present between these Z-bodies. Actin filaments overlap in these minisarcomeres, resulting in a continuous actin pattern when stained with the fluorescent F-actin staining reagent phalloidin. In the second proposed step of myofibrillogenesis, titin and overlapping muscle myosin II filaments are recruited to premyofibrils, i.e., nascent myofibrils. Mature myofibrils are recognized by the absence of non-muscle myosin II, the alignment of muscle myosin II filaments to form A-bands, and the fusion of the Z-bodies to form Z-bands [Sanger et al., 2000, 2002]. Additional support for this model of myofibrillogenesis was recently reported for myofibrils formed in the presence of taxol, and after the washout of taxol [Siebrands et al., 2004].

It would be very useful to discover compounds that inhibit myofibrillogenesis at different stages of this predicted three-step process, i.e., freeze the process at the premyofibril stage, or at the nascent myofibril stage. We have searched the literature for any evidence of such inhibitors. In 1986, Antin et al. treated developing cultured chicken skeletal muscle cells with ethyl methane sulfonate (EMS) [Antin et al., 1986], a compound commonly used to cause mutations in animals [Epstein et al., 1974] or in many types of cell lines [Nguyen et al., 1983]. Antin et al. [1986] discovered that EMS treatment prevented the formation of myofibrils and promoted the formation of a thin, broad, multinucleated cell they termed the myosheet. Within the myosheet, they observed fibers that contained both actin and non-muscle myosin II. They called these structures stress fiber-like structures (SFLS). They suggested that these fibers served as disposable templates where muscle proteins assembled into myofibrils upon removal of EMS.

These so-called stress fibers are probably premyofibrils. This report tests this hypothesis by probing fixed cells with immunostaining directed against multiple sarcomeric proteins found both in premyofibrils and in mature myofibrils. We find that in cells treated with high concentrations of EMS (10 mM), protein content and structure are very similar to premyofibrils in the spreading tips and sides of control myotubes. Lower concentrations of EMS (5 to 7.5 mM) arrested myofibrillogenesis at the nascent myofibril stage. Since the removal of EMS led to the completion of myofibrillogenesis in these arrested cells, we also searched for chemicals similar in structure to EMS (ethyl methane sulfonate) to determine if this mutagen was inhibiting myofibrillogenesis through a non-mutational pathway. We found 2-aminoethyl methanesulfonate ($MTSEA^+$), a well-known inhibitor of the ryanodine inhibitor of skeletal muscle cells, also inhibits myofibrillogenesis at an early stage.

MATERIALS AND METHODS

Reagents

EMS (ethyl methanesulfonate) and MMTS (methyl methanethiosulfanate) were obtained from Sigma Chemicals (St. Louis, MO). $MTSEA^+$ (2-amino-methanethiosulfonate) was obtained from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). These three reagents were diluted directly in quail myotube medium [Dabiri et al., 1999].

Mouse monoclonal anti-sarcomeric-alpha-actinin IgG (clone EA-53), mouse monoclonal anti-sarcomeric tropomyosin IgG (clone CH1), mouse monoclonal anti-alpha-tubulin IgG (clone DM 1A), and mouse monoclonal anti-sarcomeric-alpha-actin (clone 5C5) were obtained from Sigma Immunochemicals (St. Louis, MO). Mouse monoclonal anti-non-muscle-myosin IIB IgM (MAB1670) was obtained from Chemicon International, (Temecula, CA). Mouse monoclonal anti-titin (9D10) IgG and mouse monoclonal anti-titin (zeugmatin; mAb 20) IgG, was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-muscle-myosin II antibody was obtained as previously reported [Rhee et al, 1994]. Rhodamine Red-X-conjugated AffiniPure goat-anti-mouse-IgG, Fluorescein (DTAF) and CY2-conjugated AffiniPure goat-anti-mouse-IgM, and Texas Red-conjugated goat anti-rabbit-IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Fluorescein-conjugated phalloidin and Alexa-Fluor-488-linked phalloidin were acquired from Molecular Probes (Eugene, OR) and rhodamine-conjugated phalloidin was purchased from Fluka (Milwaukee, WI). When diluted, antibodies were diluted in standard salts (0.1 M KCl, 0.01 M K_2PO_4, 1 mM $MgCl_2$, pH 7.0).

Cell Culture

Fertilized quail eggs were purchased from CBT Farms (Chestertown, MD). Myoblasts were isolated from the breast muscles of 9-day-old quail embryos using methods previously described [Dabiri et al., 1999]. They were plated at 1×10^5 cells/ml in quail myotube medium [Dabiri et al., 1999] in Petri dishes on cover slips or in glass-bottom culture dishes when transfected. Cells were treated with varying concentrations of EMS or $MTSEA^+$ on Day 1 in culture. Except in the case of washout experiments, the cells were fixed on Day 5 in culture. Medium for the washout experiments was changed once on Day 5, and twice on Days 6, 7, and 8.

Fixing, Immunostaining, and Transfections

Most cells were fixed at room temperature in 3.0% paraformaldehyde in sodium phosphate buffer for 15 min, and permeabilized with 0.1% IGEPAL. For anti-

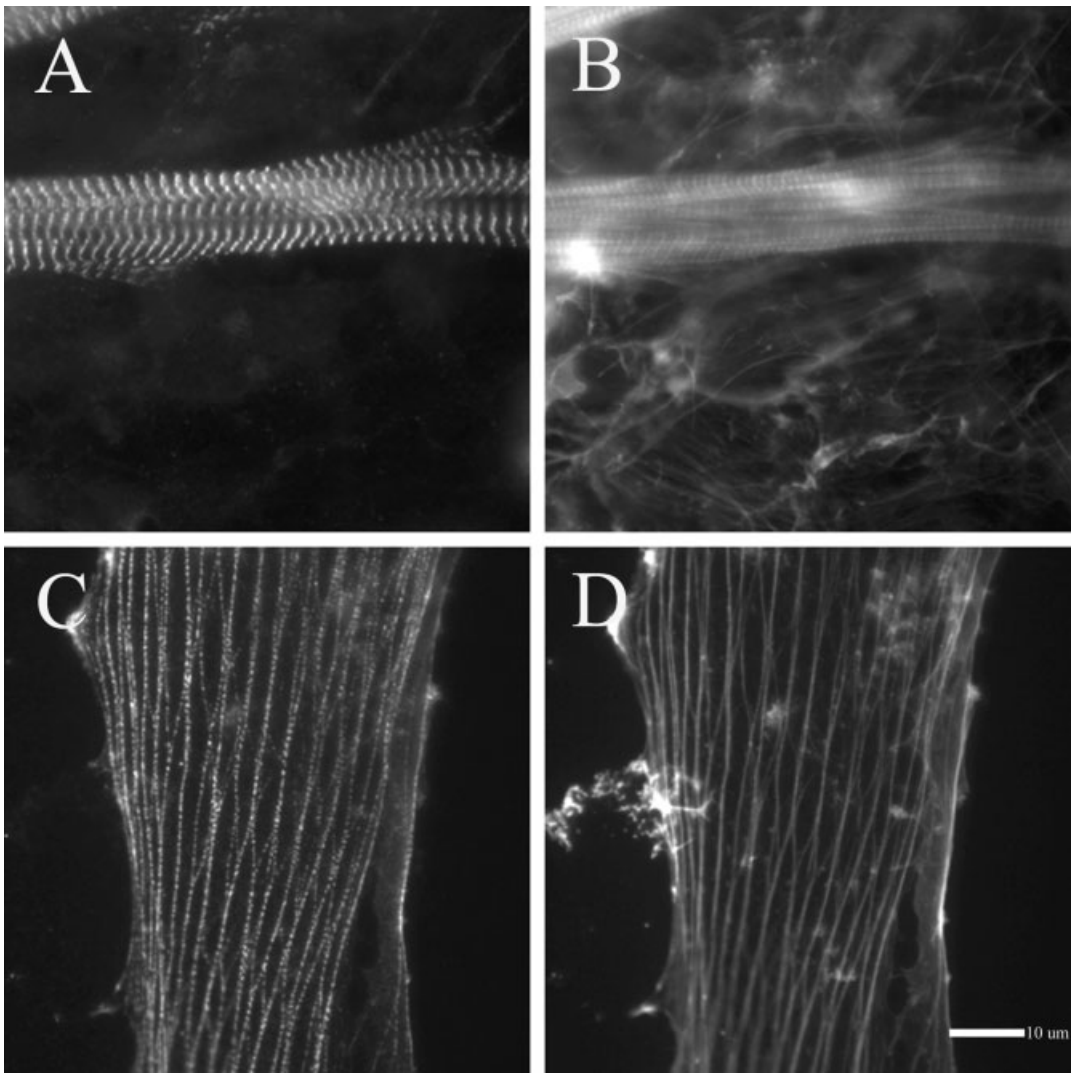

Fig. 1. Absence of mature myofibrils in quail muscle cells treated with EMS. **A,B:** Control myotubes stained with (A) anti-sarcomeric alpha-actinin or (B) phalloidin. Sarcomeres about 2 μm long are present in the control cells. The sarcomeres are bounded by the alpha-actinin enriched Z-bands (A). **C,D:** 10-mM EMS-treated cells stained with (C) anti-sarcomeric alpha-actinin or (D) phalloidin. In cells treated with 10 mM EMS, bands of sarcomeric alpha-actinin (Z-bodies) appear in a striated pattern lined up with actin. Bar = 10 μm.

tubulin staining, the cells were fixed in ice-cold 100% methanol containing 1 mM EGTA for 15 min on ice.

Cells were first stained with different primary antibodies, washed, and then incubated with rhodamine, Texas Red, or CY-2 labeled secondary antibodies. Except with anti-tubulin immunostaining, cells were counterstained with fluorescently linked phalloidin to visualize actin. When cells were immunostained with tubulin antibodies (IgGs), they were also immunostained with anti-actin antibodies (IgMs). Myotubes were incubated sequentially with primary and fluorescently linked secondary antibodies for 1 h at 37°C in humidity chambers and with fluorescently linked phalloidin for 30 min at room temperature in humidity chambers [Dabiri et al., 1999]. Cover slips were rinsed and mounted onto slides with Mowiol containing the anti-fade reagent N-propyl gallate [Dabiri et al., 1999].

Sarcomeric alpha-actinin cDNA was cut from sarcomeric alpha-actinin GFP plasmid [Dabiri et al., 1997] with HindIII and inserted into the pEYFP-N1 vector (CLONTECH, Palo Alto, CA). Myotube cultures for washout experiments were transfected with YFP-sarcomeric-alpha-actinin on Day 4 in culture with FuGENE6 reagent (Roche, Indianapolis, IN) according to the man-

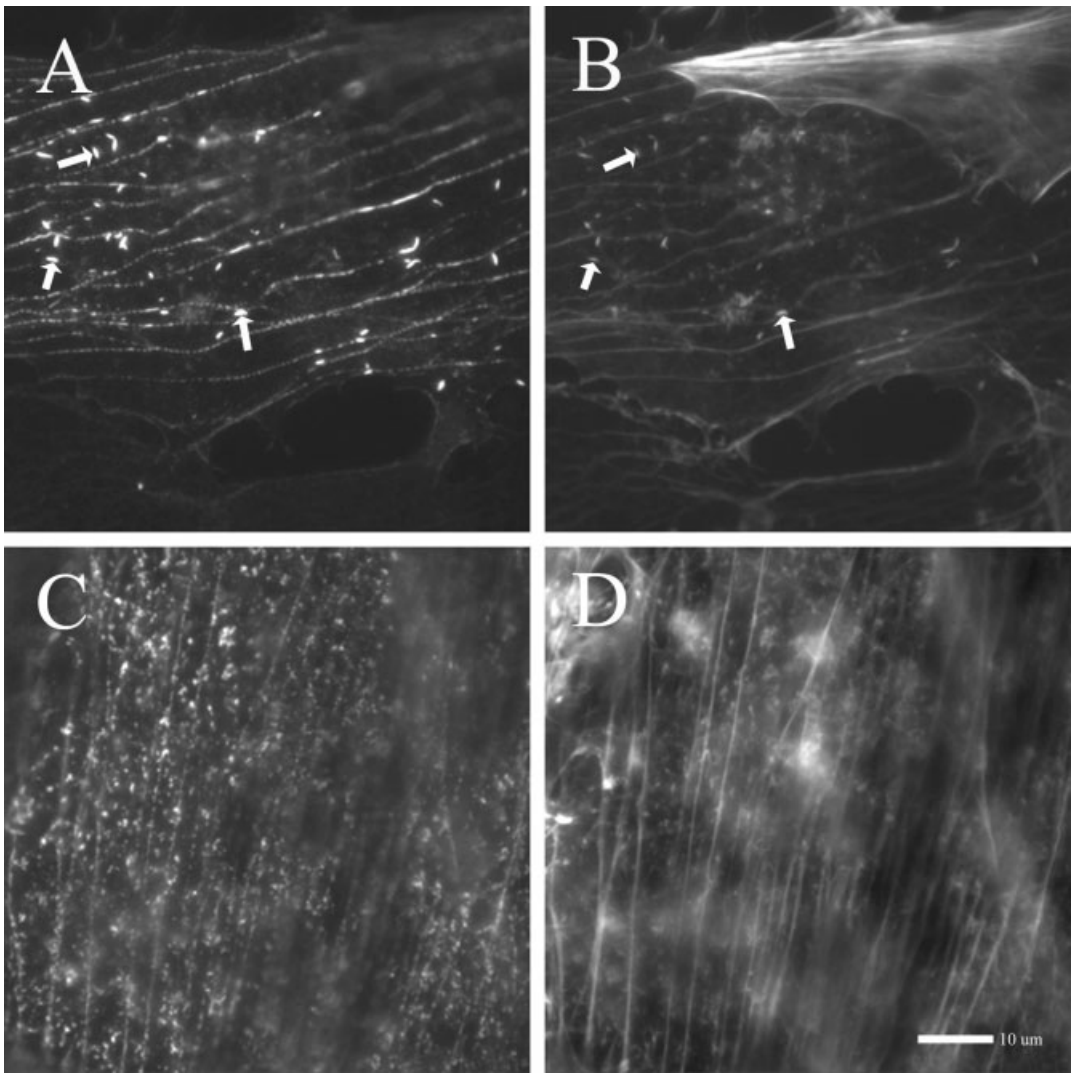

Fig. 2. Nemaline-rod-like structures (*arrows* in A, B) in quail muscle cells treated with EMS. **A:** 10-mM EMS-treated cells immunostained with sarcomeric alpha-actinin. **B:** Same microscopic field as in (A) but showing phalloidin staining. Note the fibroblast in the upper right corner in (B) that does not stain with sarcomeric alpha-actinin in A. **C:** 5-mM EMS-treated cells stained with sarcomeric alpha-actinin. **D:** Same microscopic field as in C but showing phalloidin-staining. In cells treated with EMS, rod-like protein structures containing both sarcomeric alpha-actinin and actin (nemaline bodies, *arrows* in A and B) appear randomly dispersed through cell. Size of nemaline-like bodies decreases from 10 to 5 mM EMS. Bar = 10 μm.

ufacturer's instructions. The details of these procedures have been published [Ayoob et al., 2001].

Microscopy

Images were taken with a Hamamatsu C47492-95 digital camera linked to a Nikon Diaphot 200 microscope with a Nikon Plan Fluor 100×/1.30 Oil objective. Appropriate wavelength stimulating light and filters were used to obtain images from fluorescently labeled cells. Image Pro Plus Phase 3 Imaging software was used both for acquiring pictures and for taking measurements of fluorescently labeled proteins. Measurements were taken using from 3 to 6 "sarcomeres" from the beginning of the first to the beginning of the last.

RESULTS

Distribution of Cytoskeletal Proteins in Control and EMS-Treated Cells

Skeletal muscle myoblast cultures treated with 10 and 7.5 mM EMS have a high mortality rate. The surviving myoblasts fuse with one another to form long, thin, broad, multi-nucleated sheets that do not contract;

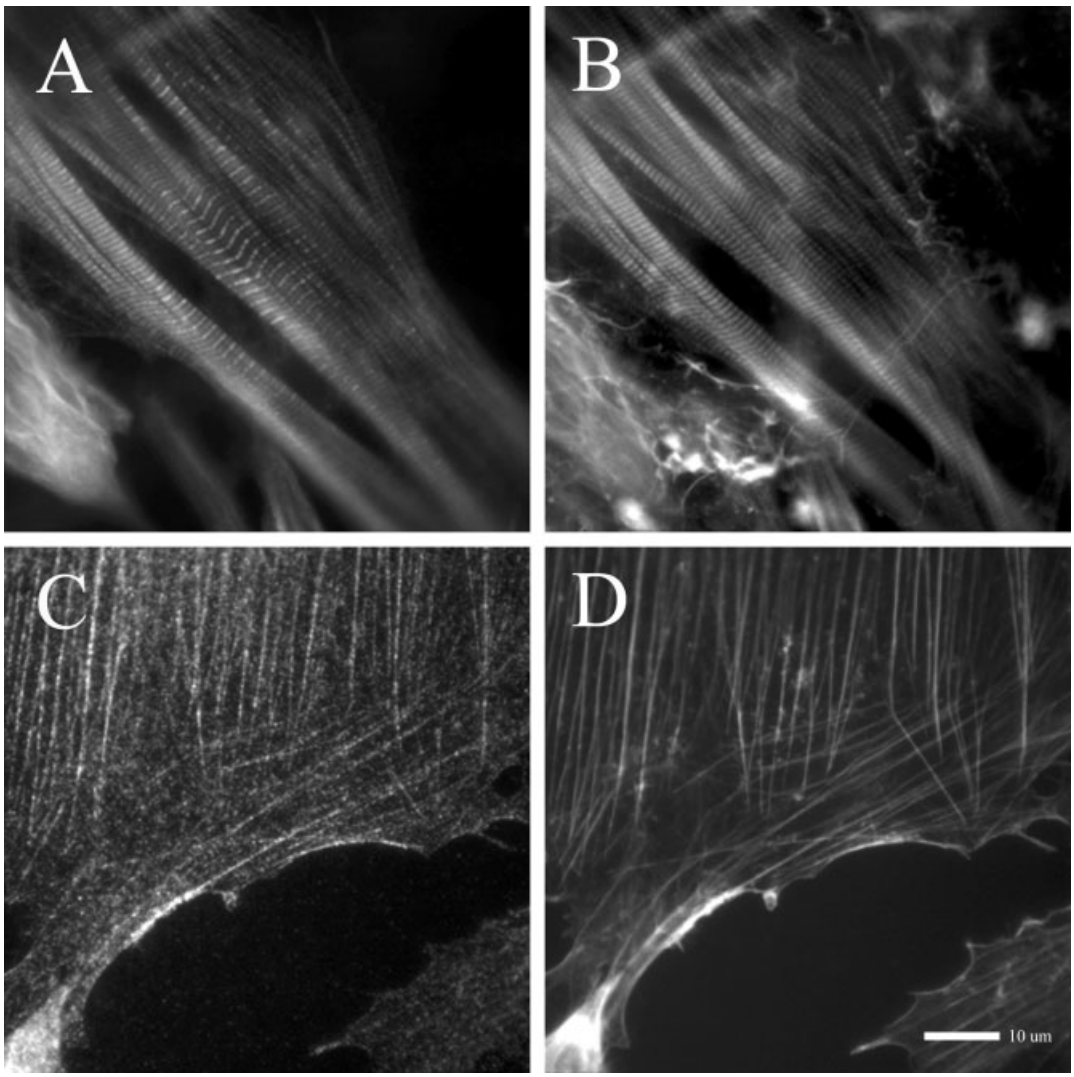

Fig. 3. **A,B:** Control myotubes stained with (A) anti-tropomyosin antibodies and (B) fluorescent phalloidin. **C,D:** Cells treated with 10 mM EMS were stained with (C) anti-tropomyosin antibodies and (D) fluorescent phalloidin. In EMS-treated cells, tropomyosin appears to be lined up with actin in a punctuate pattern, typical of premyofibrils (C, D). Bar = 10 μm.

this was first reported by Antin et al. [1986]. Cell cultures treated with 5 mM EMS have reduced cell death compared to cells in 10 or 7.5 mM EMS, but they still display fiber structures similar to 10 and 7.5 mM EMS-treated cells. EMS-treated cells (1mM) almost always appear like control cells, no matter which cytoskeletal antibody we used to probe the composition of the fibers (data not shown).

Antibodies against sarcomeric alpha-actinin in normal myotubes stain the Z-bands (Fig. 1A,B). In myosheets treated with EMS, anti-sarcomeric alpha-actinin lined up with actin and appeared in a punctate pattern (Fig. 1C,D). In normal myotubes, sarcomeres of the mature myofibrils had a length of 2.007 ± 0.193 μm (distance between two linearly aligned Z-bands); in 10 mM EMS-treated cells, average length from one Z-body to the next was 0.706 ± 0.117 μm; in 7.5-mM EMS-treated cells, the average length was 0.797 ± 0.194 μm. In fibroblasts, no sarcomeric alpha-actinin staining is seen (Fig. 2A,B).

In some myosheets, the actin and sarcomeric alpha-actinin form rod-like structures out of alignment with the actin fibers (Fig. 2). The rods appear to be randomly spaced and oriented, and can be seen in cells treated with 5–10 mM EMS. They are similar to nemaline bodies seen in nemaline rod myopathies since they contain mainly alpha-actinin and actin [see review in Karpati, 2002]. Rods are largest in 10-mM EMS-treated cells (Fig. 2A,B)

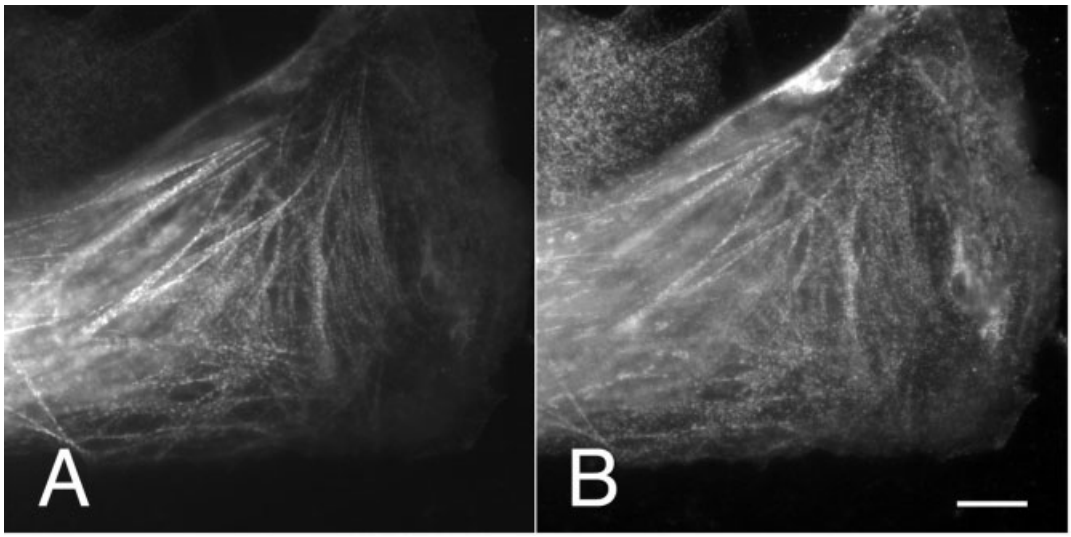

Fig. 4. The elongating end of a control quail myotube in tissue culture. **A:** Sarcomeric alpha-actinin was used to stain this area to reveal the banded patterns of the premyofibrils. **B:** The same myotube tip was stained with a non-muscle myosin IIB antibody. Sarcomeric alpha-actinin and non-muscle myosin IIB are present in the same premyofibrils. Bar = 10 μm.

and decrease in size from 10-mM EMS-treated cells to 5-mM EMS-treated cells (Fig. 2C,D). These nemaline-like bodies never appear in control myotubes.

Anti-tropomyosin antibodies stained control myotubes in a typical cross-striated pattern (Fig. 3A). A striated F-actin pattern is also detected in these control myotubes (Fig. 3B). In 10-mM EMS-treated cells, tropomyosin either appears lined up with actin in a punctate pattern (Fig. 3C,D) or diffusely through the myosheets (data not shown). Tropomyosin does not localize to nemaline-like bodies. The tropomyosin antibody we used does not stain fibroblasts.

We also probed control and EMS-treated treated cells with anti-non-muscle myosin IIB antibodies. Fine fibers staining positively for both sarcomeric alpha-actinin and non-muscle myosin IIB are present at the elongating ends of the control myotubes (Fig. 4A,B). In 10-mM EMS-treated cells, non-muscle myosin IIB lines up with actin in a beaded pattern (Fig. 5). The distance between the beads of myosin is 0.617 ± 0.085 μm. In 7.5-mM EMS-treated cells, there is much more non-muscle myosin IIB associated with individual premyofibrils than in 10-mM EMS-treated cells. Non-muscle myosin II beads are 0.669 ± 0.131 μm apart in the 7.5-mM treated myosheets. Although non-muscle myosin II was not detected in the nemaline-like bodies, it was sometimes in aggregates by itself (data not shown).

Zeugmatin is a portion of titin that is part of the Z-band [Turnacioglu et al., 1996]. Anti-zeugmatin antibodies stain the Z-bands in control myotubes (Fig. 6A). Anti-zeugmatin staining of 10-mM EMS-treated multinucleated myosheets did not reveal any fibers (Fig. 6B) that were stained with fluorescently labeled phalloidin. In addition, zeugmatin is never observed in nemaline-like bodies in 10-mM EMS-treated cells. However, in 7.5-mM EMS-treated cells**,** zeugmatin antibodies stain

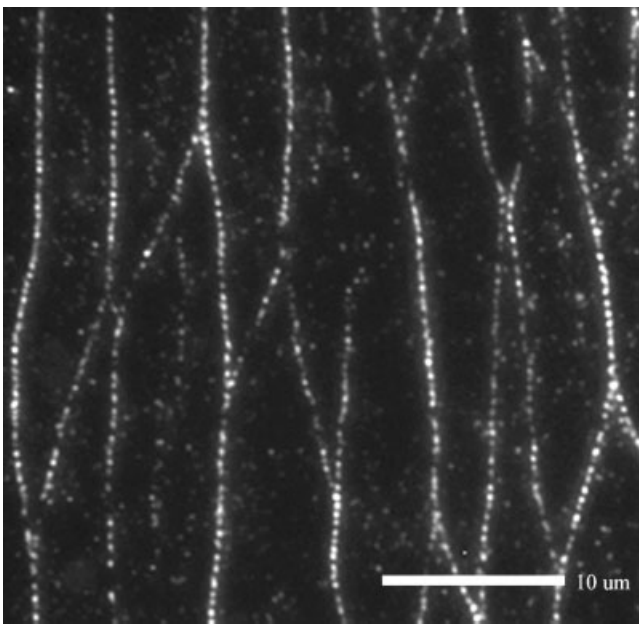

Fig. 5. EMS-treated cell was indirectly immunostained against non-muscle myosin IIB. The non-muscle myosin II is distributed along the fibers in a banded pattern previously detected in premyofibrils. Bar = 10 μm.

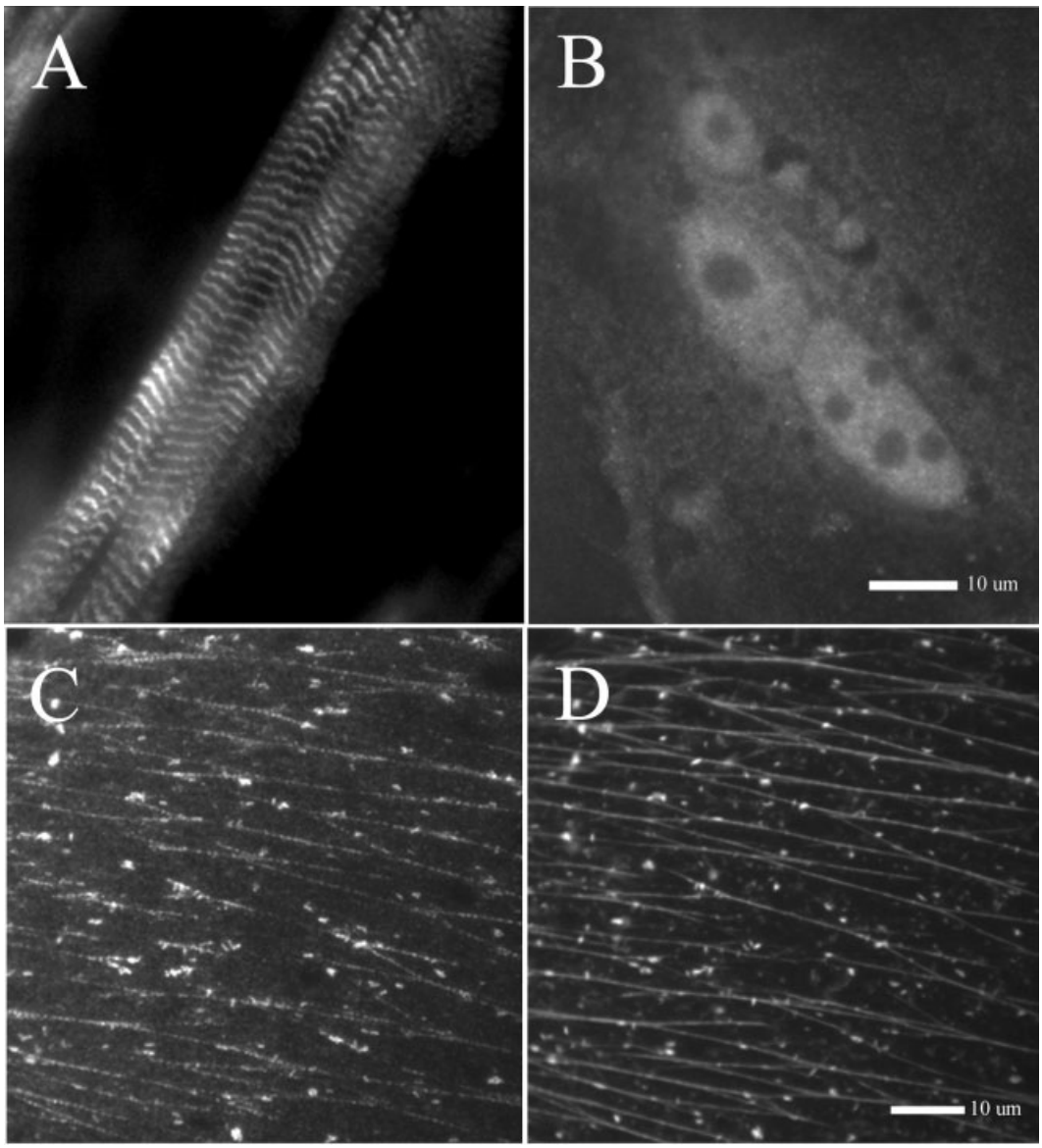

Fig. 6. (**A**) A control myotube and (**B**) a myosheet that formed in the presence of 10 mM EMS were indirectly immunostained against zeugmatin, a portion of titin that localizes to the Z-band in control cells (A). Zeugmatin stains only diffusely in 10-mM EMS-treated myosheets (B). **C,D:** Staining with the same titin antibody (zeugmatin) in 7.5-mM EMS-treated quail muscle cells (C). The myosheets were also then stained for F-actin with fluorescently labeled phalloidin (D). Unlike cells treated with 10 mM EMS, zeugmatin staining (C) shows alignment of titin with F-actin (D). Zeugmatin also localizes to rod-like structures in 7.5-mM EMS-treated cells (C, D). Bars = 10 μm.

some of the actin fibers (Fig. 6C,D). In these myosheets, the zeugmatin staining often appears beaded along part of the F-actin fibers (Fig. 6C,D). Zeugmatin often appears in the nemaline-like bodies in these 7.5-mM EMS-treated cells. The fibroblasts in these anti-zeugmatin stained cultures exhibit a diffuse cytoplasmic background and no zeugmatin is detected associated with the fluorescent phalloidin actin stress fibers (data not shown).

Anti-titin 9D10 stains titin at the A-I junctions of the sarcomeres. In control myotubes, 9D10 appears as a doublet in a striated pattern (Fig. 7A,B). In almost all 10-mM EMS-treated cells, anti-titin 9D10, like the zeugmatin titin antibody, stains diffusely throughout the cytoplasm and brightly at the nuclei (data not shown). In a few rare 10-mM EMS-treated myosheets, 9D10 sometimes aligns with actin and appears in an irregularly spaced punctate pattern (Fig. 7C,D). In 7.5-mM EMS-treated cells, 9D10 aligns with actin in an irregularly spaced punctate pattern (Fig. 8A,B). Anti-titin 9D10 does not stain fibroblasts.

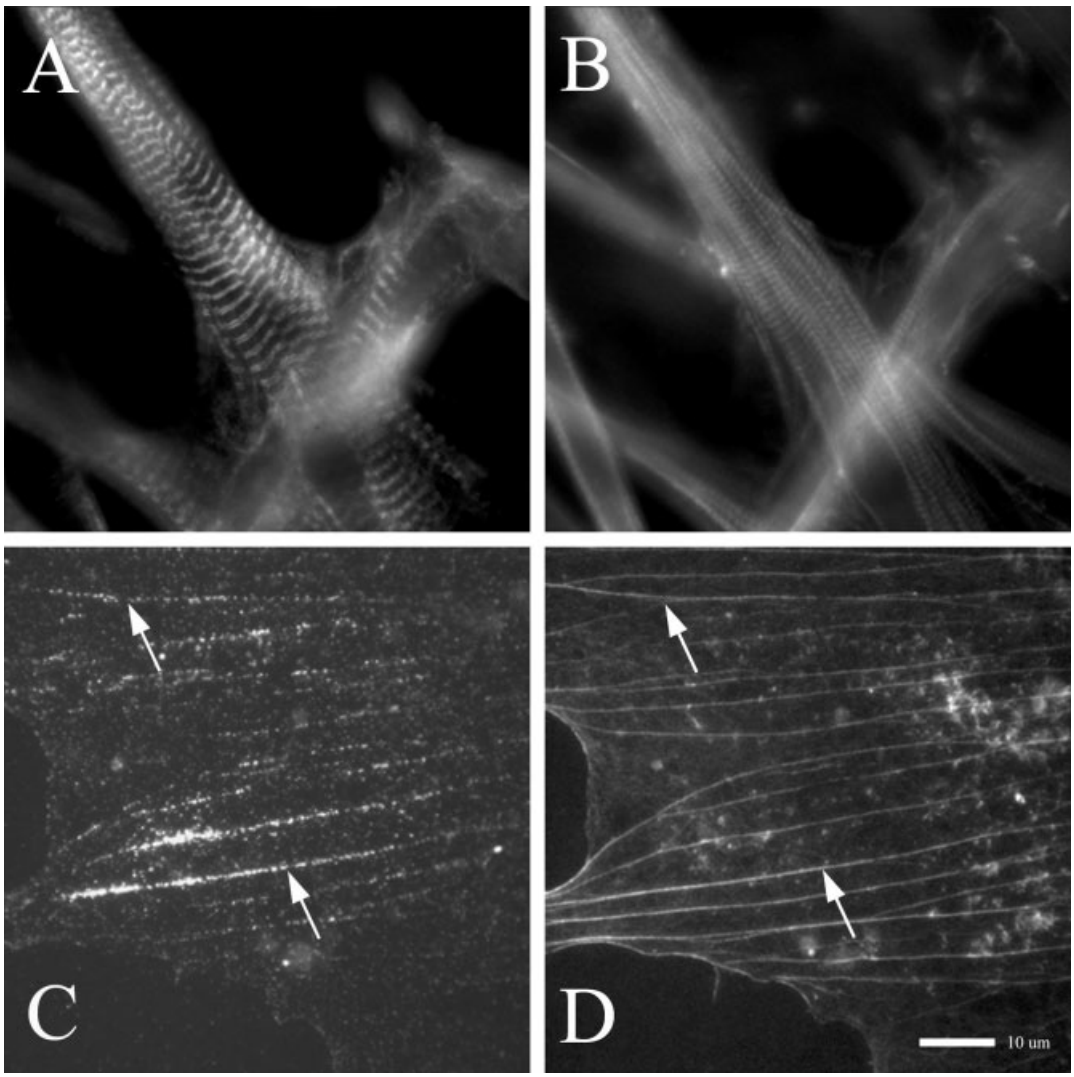

Fig. 7. Control myotubes were stained with (**A**) a titin antibody (9D10) that stains sarcomeres at the junction of the A-I bands and (**B**) fluorescent phalloidin. **C, D:** A quail myosheet was indirectly immunostained with (C) anti-titin 9D10 antibody, and (D) fluorescent phalloidin. In most 10-mM EMS-treated myosheets, anti-titin 9D10 stained diffusely throughout the cytoplasm as shown in Figure 6B. In a few myosheets formed in the presence of 10 mM EMS, some of the actin fibers (*arrows* in D) stain positively for this particular titin antibody (*arrows* in C). These actin fibers exhibit short-banded patterns of titin that are equivalent to those spacings of alpha-actinin and non-muscle myosin IIB in premyofibrils and nascent myofibrils. Bar = 10 μm.

In control myotubes, muscle myosin II is organized into A-bands that align across muscle fibers (Fig. 9A). In the tips of control myotubes (Fig. 9B–D), unstriated arrays of muscle myosin II and diffuse myosin can be seen (Fig. 9B,D). In the elongating ends of control myotubes, two types of actin fibers are detected: actin fibers unassociated with muscle myosin II antibodies (premyofibrils, Fig. 9C,D); actin fibers associated with unbanded or unstriated arrays of muscle myosin II (nascent myofibrils, Fig. 9C,D). In some cells treated with 10 mM EMS, muscle myosin II is oriented in the same direction as some of the actin fibers (Fig. 10A,B). In most other cells in this treatment group, muscle myosin II is spread diffusely through the cell. In cells treated with 7.5 mM EMS, muscle myosin II always aligns with actin (Fig. 10C,D). Visually, the level of muscle myosin is greatly reduced in myosheets compared to control myotubes. Our anti-muscle myosin II antibody never stains fibroblasts (data not shown).

In the few 10-mM EMS-treated cells in which titin 9D10 aligned with actin fibers, muscle myosin II also co-localized with these fibers. In many cells, although both proteins were visible, titin did not align with the actin fibers (data not shown). When cells were probed

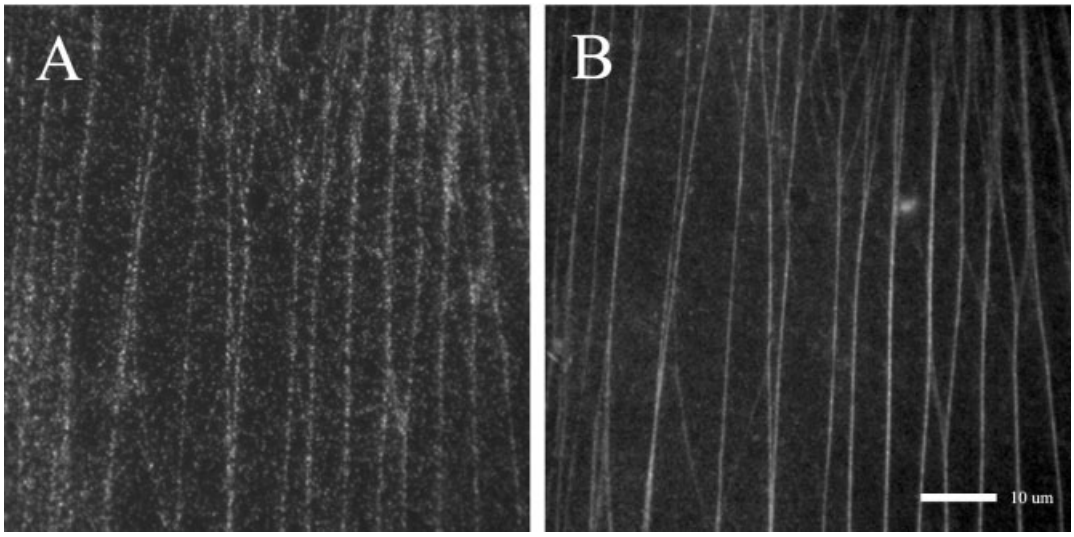

Fig. 8. In the presence of 7.5 mM EMS all of the fibers present in the myosheets stained positively for the (**A**) 9D10 titin antibody (9D10) and (**B**) fluorescently labeled phalloidin. The short spacings of titin in A were identical to those detected in myosheets formed in the presence of 7.5 mM EMS and stained with the zeugmatin anti titin antibody (see Fig. 6C). Bar = 10 μm.

with both anti-muscle myosin II and with anti-titin-9D10, the 9D10 portion of titin and muscle myosin always co-localized in 7.5-mM EMS-treated cells (data not shown).

To determine whether all cytoskeletal proteins were affected by EMS, we probed skeletal muscle cells with anti-tubulin antibodies. We counterstained them with an anti-actin antibody. The presence and distribution of microtubules were not affected by treatment with EMS (data not shown).

Antin et al. [1986] reported that myosheets recovered completely after exposure to EMS. We transiently transfected cultured cells on Day 4 with sarcomeric-alpha-actinin-YFP and then started washout experiments with normal medium on Day 5. On the first day after washout, treated myotubes showed little recovery (Fig. 11A). Sarcomeric alpha-actinin still lined up with actin, and nemaline-like rods could be observed. By Day 10 in culture, all cells still expressing the fluorescent plasmid had completely recovered and had normal Z-bands (Fig. 11B). The nemaline-like bodies had disappeared (Fig. 11B). On Day 8, some myotubes had completely recovered, but some were still in the process of recovering. By this time, some myotubes were contracting. We followed live transfected cells that had not yet recovered from EMS exposure over the course of Day 8. We observed fibers containing sarcomeric alpha-actinin Z-bodies (Fig 11C). Over the next 2 h, the Z-bodies aligned and fused to form Z-bands (Fig. 11D). A contraction occurred shortly before the image for Figure 11D was taken.

Derivatives of EMS

Methyl methanethiosulfanate (MMTS) and 2-aminomethanethiosulfonate (MTSEA$^+$ are derivatives of EMS (Fig. 12). Both compounds (i.e., MMTS and MTSEA$^+$) have been used as ion channel blockers [Akabas et al., 1992]; in particular, they have been used to inactivate the ryanodine receptor [Quinn and Ehrlich, 1997]. At 5 mM, MTSEA$^+$ completely blocked the ryanodine receptor and MMTS blocked the ryanodine receptor current by half. In our experiments, application of 10, 5, and 1 mM MMTS was toxic to all cells. At 0.1 mM, no effects were seen in developing myotubes. Application of 7.5 and 10 mM MTSEA$^+$ to quail skeletal muscle cells was toxic to all cells. At 5 mM MTSEA$^+$, 5-day cultures fixed and stained with anti-sarcomeric alpha-actinin exhibited no Z-bands. Instead, sarcomeric alpha-actinin was detected in Z-bodies that lined up with actin fibers just as in cells treated with EMS (Fig. 13A,B). In some cases, nemaline-like bodies formed (Fig. 13C,D). These nemaline-like bodies stained positively for both sarcomeric alpha-actinin and F-actin. The 5-mM MTSEA$^+$-treated cells were multi-nucleated and broad but not flat. After 5 days in culture, many of the multi-nucleated cells had grown in length as well as by fusing. In contrast to 10-mM EMS-treated cells, there were many myoblasts remaining in culture that had not fused. These unfused cells die in EMS-treated cultures. Some effects were seen even in 1-mM MTSEA$^+$ -treated cells. These cells had broad, long tips, but sarcomeric alpha-actinin was expressed in normal Z-band patterns in the middle of the myotubes,

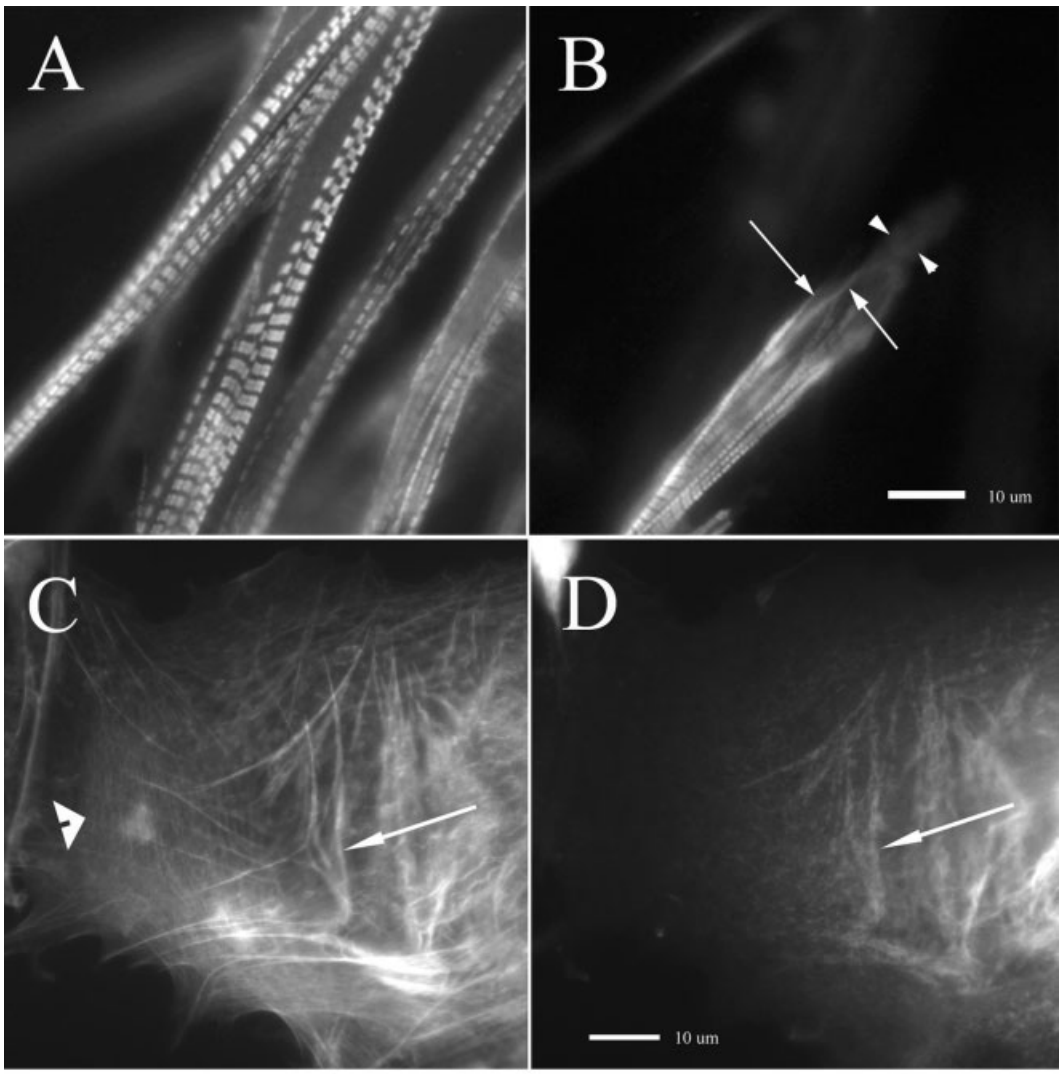

Fig. 9. Muscle myosin II in control myotubes. Control quail myotubes were indirectly immunostained with an anti muscle myosin II antibody. **A:** Muscle myosin II appears in A-bands in mature myofibrils. **B:** The end of a myotube reveals three different patterns of muscle myosin II localization: A-bands typical of mature myofibrils; continuous patterns of myosin (*arrows*) typical of nascent myofibrils; background levels of staining (*arrowheads*) representing no myosin or at least no organized muscle myosin II at the very end of the myotube, i.e., premyofibrils. (**C**) F-actin and (**D**) muscle myosin II at the end of a myotube. (C) The *arrowhead* points out actin fibers unassociated with muscle myosin in D. These are premyofibrils. The *arrows* in C, D indicate actin fibers (C) that are associated with bundles of unstriated muscle myosin filaments (D), i.e., nascent myofibrils. Bars = 10 μm (A and B same magnification; C and D same magnification).

and the myotubes were of a normal width in the middle. After the 5-mM $MTSEA^+$ was removed from the cultures, normal mature myofibrils formed after several days.

DISCUSSION

The proteins present and their localization in EMS-treated cells suggest that the myosheets are arrested in normal muscle development at the premyofibril and nascent myofibril stages. In 10-mM EMS cultures, the fibers that assemble in myosheets contain the same proteins in the same localization as premyofibrils (sarcomeric alpha-actinin, tropomyosin, F-actin, and non-muscle myosin IIB). The fibers that form in the myosheets in 7.5-mM cultures contain the same proteins as nascent myofibrils (sarcomeric alpha-actinin, tropomyosin, F-actin, and non-muscle myosin IIB; titin, and arrays of muscle myosin II) [Rhee et al., 1994; Sanger et al., 2002; Du et al., 2003a,b]. Sarcomeric alpha-actinin, non-muscle myosin IIB, muscle myosin II, actin, tropomyosin, and the A-I portion of

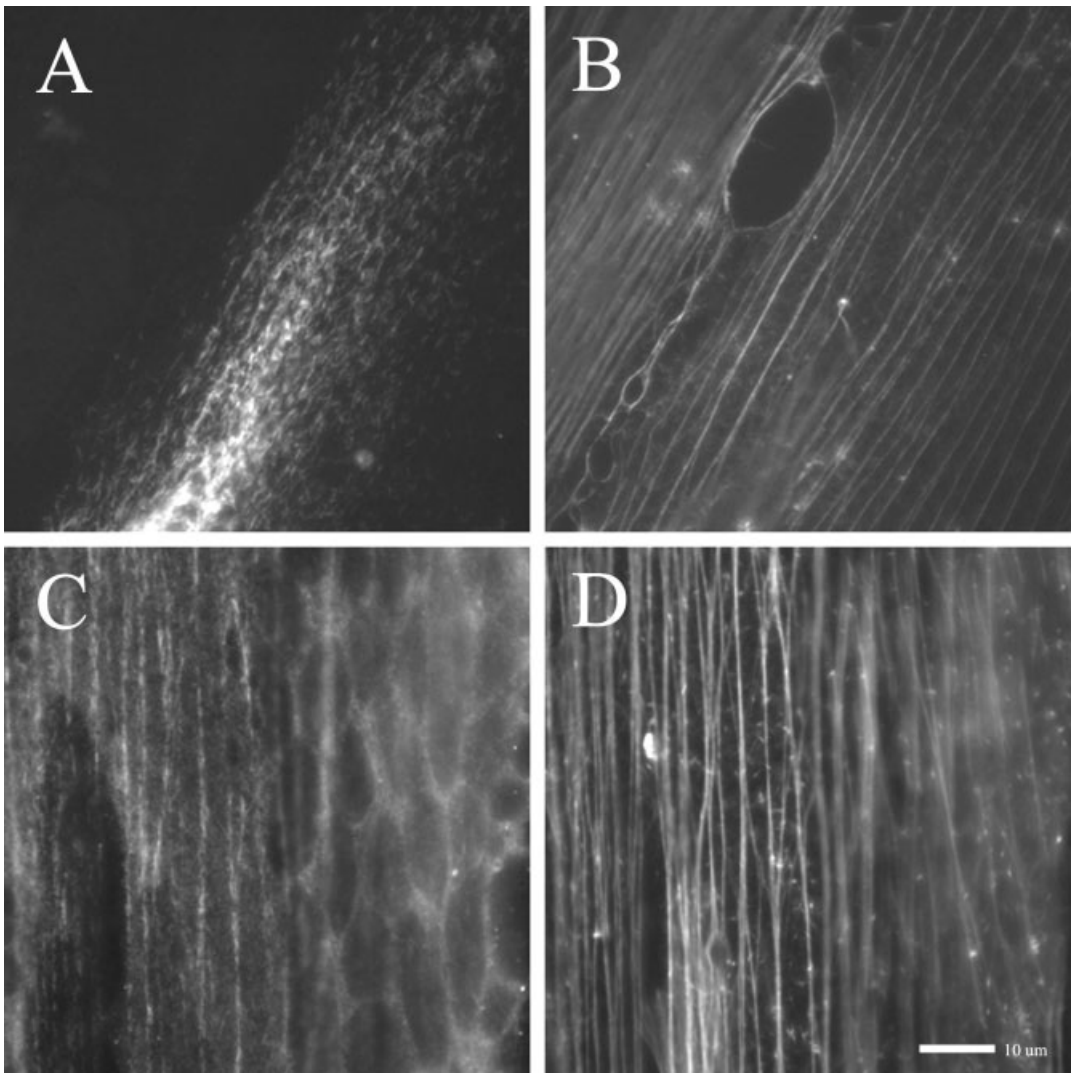

Fig. 10. Muscle myosin II loses its organization in cells treated with EMS. Cells were indirectly fluorescently stained with a specific muscle myosin II antibody (A, C) or with phalloidin (B, D). **A,B:** Same microscopic field of 10-mM EMS-treated cells. No A-bands are apparent. Muscle myosin runs in the same direction as actin, but not always along actin filaments. **C,D:** In 7.5-mM EMS-treated cells, muscle myosin II always colocalizes with actin. Bar = 10 μm.

titin all appear in 7.5- and 10-mM EMS-treated cells much as they appear in the tips of untreated myotubes at early stages of development [Sanger et al, 2002, Siebrands et al., 2004]. Removal of the inhibitor permitted mature myofibrils to form. Our results add further support to a three-step model of myofibrillogenesis: premyofibril to nascent myofibril to mature myofibril.

We suspect that the arresting effects of EMS and the EMS derivative MTSEA$^+$ can be attributed to the inactivation of ryanodine receptors. EMS derivatives are widely used for blocking ion channels. They are thought to block ion channels by binding sulfhydryl containing groups within the ion channel pore [Deng et al., 2000; Quinn and Ehrlich, 1997]. Quinn and Ehrlich [1997] report that 5 mM MTSEA$^+$ completely blocks the ryanodine receptor in patch-clamp experiments. Since EMS and MTSEA$^+$ are similar in structure, EMS may also block the ryanodine receptor. Monnier et al. [2000] and Scacheri et al. [2000] report separate spontaneous human ryanodine receptor mutations that cause central core disease as well as nemaline rod myopathy.

Nemaline myopathies are human diseases that result in muscle weakness and are diagnosed by threadlike rods in biopsies of muscle [Karpati, 2002]. These rods usually contain actin and alpha-actinin but have been

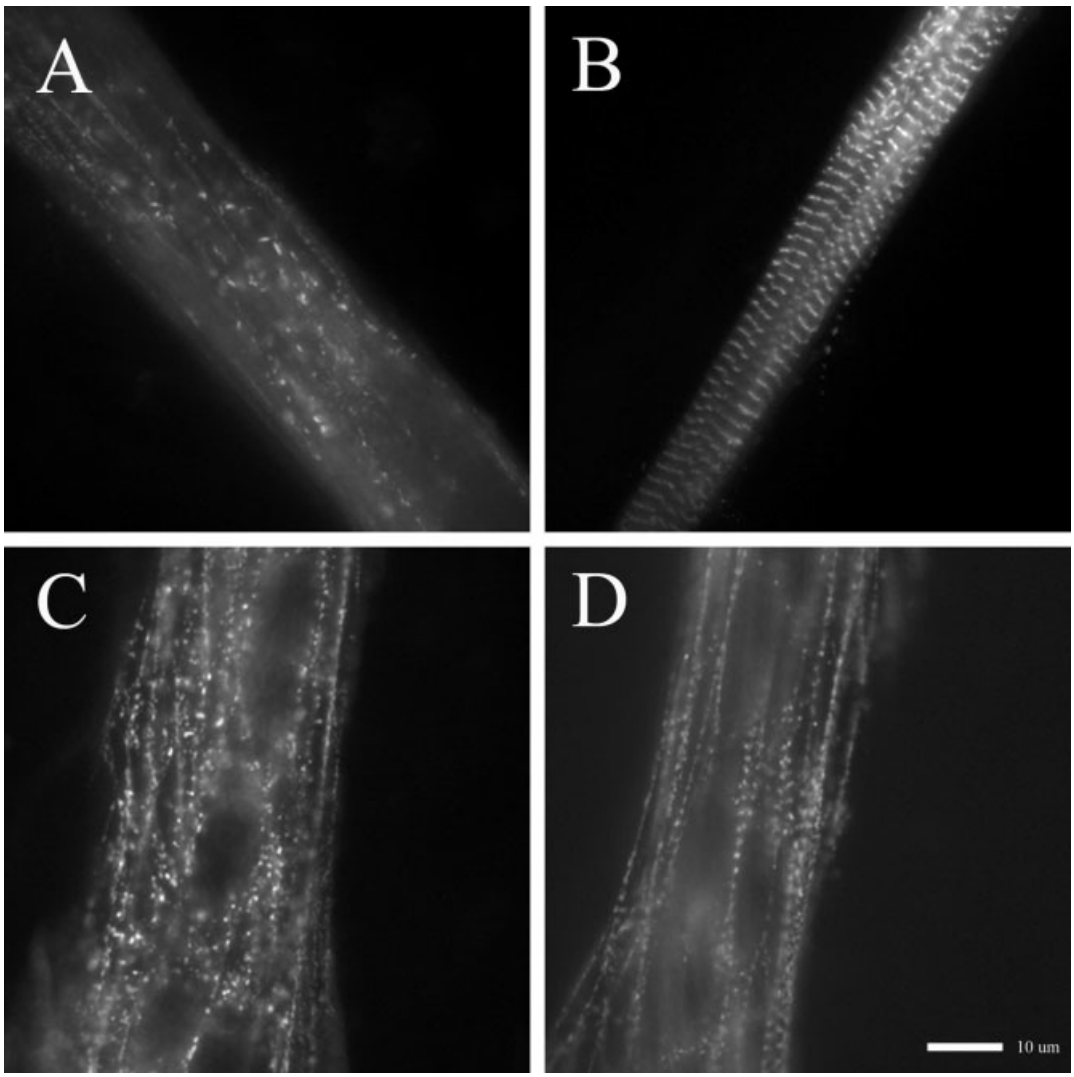

Fig. 11. Complete recovery of EMS-treated myotubes after washout of the inhibitor. EMS-treated quail skeletal muscle cells (5 mM) were transfected with sarcomeric-alpha-actinin-YFP before washout. **A:** Cells expressing sarcomeric-alpha-actinin-YFP one day after washout show very little recovery. **B:** Quail myotube expressing sarcomeric alpha-actinin-YFP 5 days after washout of EMS appears identical to control myotubes transfected with plasmids encoding sarcomeric alpha-actinin-YFP. **C,D:** The same live myotube expressing sarcomeric-alpha-actinin-YFP 2 h apart on day 3 after washout of the EMS. Premyofibrils in C have fused to form mature myofibrils in D. Bar = 10 μm.

shown to contain other muscle proteins such as vinculin and vimentin [van der Ven et al., 1995]. Mutations in a variety of muscle proteins such as muscle myosin II [van der Ven et al., 1995], actin, nebulin, tropomyosin, and troponin [Ryan et al., 2001] have been reported to cause nemaline myopathies.

Monnier et al. [2000] reported a mutated ryanodine receptor with abnormal calcium release. This mutation [Scacheri et al., 2000] causes a misfolding of the ryanodine receptor, which leads to aggregate formation in the cytoplasm and prevents insertion of the protein into the sarcolemmic membrane. Li et al. [2002] report a Ca^{2+}-dependent checkpoint during cardiac myofibrillogenesis. When calcium was not present, mRNA and protein levels of cardiac myosin light chain MLC2v were drastically reduced, the MLC2v present was not phosphorylated, and alpha-actinin and MLC2v were not correctly inserted into sarcomeres. Blockage of ryanodine receptors would disrupt flow of calcium into the cytoplasm of the EMS or $MTSEA^+$ treated cells and could lead to improper insertion of sarcomeric alpha-actinin and muscle myosin II and to the formation of nemaline-like bodies.

However, the view that reduced calcium levels lead to the phenotype we observe in our treated cells may be

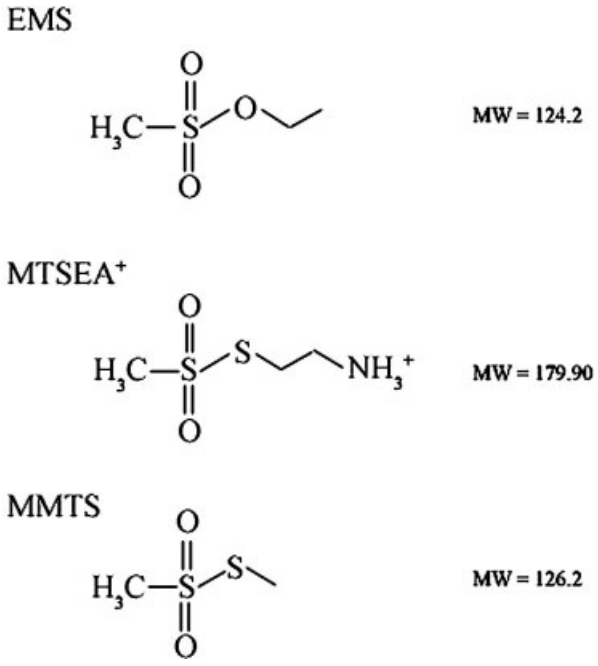

Fig. 12. Chemical formulae of EMS, MTSEA+ , and MMTS.

an oversimplification. A mutation in the ryanodine receptor of one family with central core disease causes higher-than-normal levels of Ca^{2+} in the cytoplasm of resting cells and reduced levels of Ca^{2+} current in cells stimulated with caffeine [Lynch et al., 1999]. Both sequestering and release of Ca^{2+} seem to be important in myofibrillogenesis.

We treated cultured skeletal muscle cells with $MTSEA^+$ after seeing the effects of EMS. We indirectly immunostained the fixed cells with rhodamine-anti-sarcomeric alpha-actinin. The two reagents (EMS and $MTSEA^+$) caused similar effects. They both caused broadening of the myotubes, nemaline-like bodies, and the loss of long spacings of alpha-actinin, i.e., Z-bands, within the myotubes. However, treatment with 5 mM $MTSEA^+$ did not result in the extremely thin myosheets that treatment with EMS does. Different effects caused by the two reagents may result from different secondary inhibitory effects of the two reagents. Concentrations required for either compound to affect the myotubes are higher than normally desired for use with an inhibitor. At these millimolar concentrations, both compounds may have inhibitory effects on other cellular processes. At a minimum, we know that $MTSEA^+$ blocks multiple ion channels, including calcium-activated potassium channels in smooth muscle [Wang et al., 1997], sodium channels in cardiac muscle, [Sunami et al., 2000], and the acetylcholine receptor channel [Akabas et al., 1992]. EMS is also a known oxidizing reagent that can cause cell death through lipid peroxidation [Termenstein, et al., 2000]. However, fibroblasts grown in culture along with the quail skeletal muscle are not affected when treated with 10 mM EMS. No nemaline-like bodies or complete loss of stress fibers is ever seen in fibroblasts, so we believe that these effects are muscle-specific. Since fibroblasts do not have ryanodine receptors, the muscle-specific effects we see may be mediated through the ryanodine receptor.

Antin et al. [1986] discussed the development of so-called stress-fiber-like structures into myofibrils upon reversal of EMS. They reported that the treated cells before recovery were essentially the same as fibroblasts in their protein content. Our immunofluorescence studies utilizing antibodies that have been developed since the first report of the effects of EMS on skeletal muscle cells were able to show many differences between fibroblasts and EMS-treated skeletal muscle cells, i.e., myosheets. Sarcomeric muscle myosin II, sarcomeric alpha-actinin, tropomyosin, and titin do not appear in fibroblasts. Furthermore, our studies of live cells recovering from the effects of EMS demonstrate that premyofibrils fuse to form mature myofibrils (Fig. 11). Similar fusions of premyofibrils were observed in living cardiomyocytes also transfected with GFP-alpha-actinin [Dabiri et al., 1997]. These results in this report and by Dabiri et al. [1997] are inconsistent with the stress fiber as a disposable template on which a mature myofibril is assembled from different parts of a sarcomere.

Although Antin et al [1986] report that chick myotubes treated with EMS contain no muscle myosin, we clearly see some muscle myosin in our quail myotubes probed with anti-muscle myosin II antibody. This difference in our results may stem from differences in the two species used for cell culture; we used quail and they used chicken. In their EM studies, they show some muscle myosin in recovering myosheets but not in treated cells. We did note greatly reduced levels of muscle myosin II in EMS-treated cells.

Titin has been proposed as a molecule that targets muscle myosin II to the sarcomere and is thought to be necessary in sarcomere assembly [Rhee et al., 1994; Clark et al., 2002]. In EMS-treated cells, not only are muscle myosin II levels greatly reduced, but also the muscle myosin that is present is not inserted correctly. No mature sarcomeric structures are present in 7.5- to 10-mM EMS-treated cells. Sarcomeric alpha-actinin deposits arranged in Z-bands are not present in the 5- to 10-mM EMS treated cells. Sarcomeric alpha-actinin is present in Z-bodies, and the distance between them is similar to the spacings of Z-bodies in premyofibrils [Rhee et al., 1994; Du et al., 2003a]. Premyofibrils do not stain for titin [Rhee et al., 1994; Du et al., 2003a]. In the presence of 10-mM EMS, no zeugmatin (Z-band region of titin) antibody staining was detected in the actin fibers

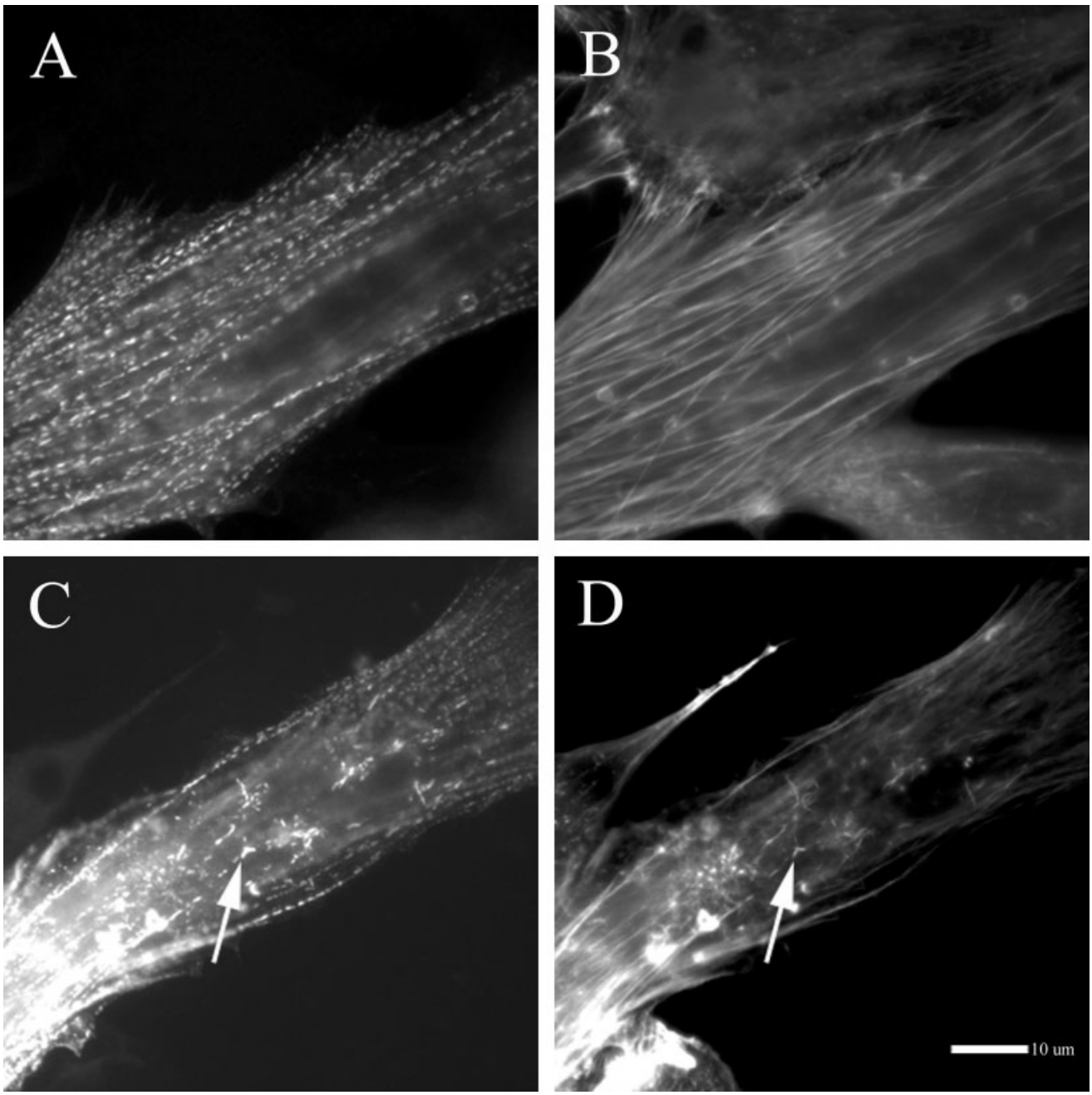

Fig. 13. $MTSEA^+$ also arrests development of myofibrils. Myoblast cultures were treated with 5 mM $MTSEA^+$ and stained with (**A**) anti-sarcomeric alpha-actinin and (**B**) counterstained with fluorescent phalloidin. **C,D:** Nemaline-like bodies appeared in these cultures as well as those treated with 5–10 mM EMS. (C) Anti-sarcomeric alpha-actinin antibodies and (D) phalloidin stained the nemaline-like bodies (*arrows*) and fibers in these cells. Bar = 10 μm.

present in the myosheets. These F-actin fibers also stained for sarcomeric alpha-actinin, tropomyosin, and non-muscle myosin IIB, just like the premyofibrils of control myotubes and cardiac muscle cells [Rhee et al., 1994; Sanger et al., 2002; Siebrands et al., 2004]. The A-I region of titin shows little association with actin fibers in 10-mM EMS-treated cells, and muscle myosin II is rarely present along actin fibers. In the few cases when the A-I region of titin is present in some of the actin fibers in the 10-mM EMS-treated cells, muscle myosin II was also localized along those actin fibers. In 7.5-mM EMS-treated cells, in which the A-I portion of titin is

always lined up with actin, and in which the zeugmatin region of titin is sometimes lined up with F-actin, muscle myosin II and the A-I portion of titin always co-localize. In 5-mM EMS-treated cells, both portions of titin line up with the F-actin fibers. Non-striated arrays of muscle myosin II aligned with actin fibers in these myosheets. These non-striated muscle myosin II-actin fibers also stain with antibodies directed against sarcomeric alpha-actinin, tropomyosin, and non-muscle myosin IIB; properties similar to nascent myofibrils in control muscle cells [Rhee et al., 1994; Sanger et al., 2002; Siebrands et al., 2004]. These correlations suggest, but do not prove, that titin is required for recruitment of muscle myosin II filaments into the nascent myofibril; however, the correlations do indicate that the insertion of both titin and muscle myosin II requires some calcium dependent factor, and that the recruitment of titin and muscle myosin II occurs almost simultaneously.

Even though we used a reagent that is known to block ryanodine receptors, MTSEA$^+$, and saw similar results in cells treated with this reagent and EMS, we still cannot be certain that EMS itself blocks ryanodine receptors or if inhibition of the ryanodine receptor is the only factor causing the morphology of treated cells. Patch-clamp experiments could determine whether EMS blocks ryanodine receptors. We could also use experiments with calcium to determine its direct effects on the morphology of developing muscle cells. Another ryanodine receptor inhibitor, methyl methanethiosulfanate (MMTS), which is used in short-time exposures on intact muscle cells, proved to be toxic at almost all concentrations on myoblasts.

SUMMARY

There is now increasing evidence that myofibril assembly follows the same three-step process proposed by Rhee et al. [1994] in cardiac and skeletal muscle cells in tissue culture [Sanger et al., 2000, 2002; Sanger and Sanger, 2002; Siebrands et al., 2004], in precardiac explants [Du et al., 2003a], and, now, even in cardiac muscle cells in early intact embryonic hearts [Du et al., 2003b]. We believe that studies with EMS or MTSEA$^+$ may help elucidate the process of myofibrillogenesis. Halting the development at the premyofibril stage and nascent myofibril stage and the slow reversal of these reagents in combination with transfections using GFP plasmids encoding different sarcomeric proteins, and immunofluorescence assays, may be able to demonstrate the order of protein assembly in myofibrillogenesis, and identify which components are responsible for the process.

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