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Studies on the Function of Rho A Protein in Cardiac Myofibrillogenesis

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Abstract The aim of this study was to provide morphological evidence for the presence of rho A protein in developing cardiomyocytes and to investigate its possible role in myofibrillogenesis. Immunostaining with a monoclonal anti-rho antibody gave a diffuse pattern in the cytosol of cultured cardiomyocytes. Introduction of C3 exoenzyme into the cells by electroporation was used to inactivate rho A protein by ADP-ribosylation. An immunostaining with anti-vinculin, anti-talin, and anti-integrin antibodies showed the focal adhesions in electroporation control cardiomyocytes to be evenly distributed in the ventral sarcolemma; the costameric structure was also detected using these antibodies. In contrast, in C3 exoenzyme treated cells, focal adhesions were disassembled and costamere were absent; in addition, β-actin-positive, non-striated fibrils were lost and assembly of M-protein, titin, and α-actinin into myofibrils was poor, as shown by diffuse and filamentous staining pattern. C3 exoenzyme treatment had a less marked effect on mature cardiomyocytes than on immature cells; in this case, cells became distorted and few myofibrils were seen. The intensity of anti-phosphotyrosine antibody staining of the focal adhesion was also decreased or diffuse in C3 exoenzyme-treated cardiomyocytes, suggesting dephosphorylation of focal adhesion components. We therefore conclude that small G protein rho A plays an important role in myofibril assembly in cardiomyocytes. J. Cell. Biochem. 66:43–53, 1997.

Key words: rho A; C3 exoenzyme; focal adhesion; costamere; myofibrillogenesis; cardiomyocyte

The actin cytoskeleton in non-muscle cells is organized into the cortical actin network, stress fibers, and cell surface protrusions [Small, 1988]. Stress fibers are connected to the membrane-extracellular matrix complex via focal adhesions (FAs) composed of cytoplasmic components, including vinculin, talin, tensin, α-actinin, paxillin and focal adhesion kinase, and a transmembranous protein, integrin. FAs act as a linker between the actin cytoskeleton and the extracellular matrix and are sites of signal transduction [Burridge et al., 1988; Turner and Burridge, 1991]. The formation of FA and stress fibers is dependent on rho A protein, a member of the small G proteins [Paterson et al., 1990; Ridley and Hall, 1992]. The biological function of Rho proteins can be inactivated by C3 exoenzyme (C3) via ADP-ribosylation on amino acid Asn-41 of the Rho protein [Aktories et al., 1989]. Microinjection of C3 into a variety of cell types results in the loss of stress fibers and FA [Rubin et al., 1988; Chardin et al., 1989], while the introduction of recombinant rho protein or plasmids expressing rho cDNAs into serum-starved quiescent fibroblasts re-establishes the organization of the actin cytoskeleton [Paterson et al., 1990]. The general assumption is that activation of rhoA stimulates tyrosine phosphorylation of pp125^{FAK}, p130, and paxillin in Swiss 3T3 fibroblasts [Burridge et al., 1992; Barry and Critchley, 1994; Hemmings et al., 1995; Flinn and Ridley, 1996], which, in turn phosphorylates several FA proteins and induces the formation of FA and stress fibers. Indeed, C3 treatment is found to inhibit tyrosine phosphorylation of p125^{FAK} and paxillin stimulated by GTPγS [Seckl et al., 1995] or sphingosyl phosphoryl-choline [Seufferlein and Rosengurt, 1995] in Swis 3T3 fibroblasts.

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Most of the above-mentioned studies on rho A, one of the main cellular rho proteins, involved non-muscle cells. In smooth muscles, C3 treatment abolishes GTPγS-induced calcium sensitization, suggesting that rho A is involved in the calcium-regulated contraction of smooth muscle [Hirata et al., 1992]. However, the precise function of rho A in cardiac and skeletal muscles is still unknown.

Developing cardiomyocytes contain many non-striated fibrils which are similar in several ways to the stress fibers of non-muscle cells [Dlugosz et al., 1984; Handel et al., 1991; Wang et al., 1988]. These non-striated fibrils are attached to the sarcolemma at the FA via their ventral tips in order to maintain their structural integrity [Lu et al., 1992]. In addition, several FA proteins, including vinculin, integrin, and talin, also form the costamere, a rib-like structure connecting the Z-lines of peripheral myofibrils to the ventral or lateral sarcolemma [Pardo et al., 1983; Terracio et al., 1990; Danowski et al., 1992]. Addition of anti-integrin β1 subunit antibody to the culture medium effectively inhibits myofibril assembly by perturbing cell adhesion in both developing chicken myotubes [McDonald et al., 1995] and neonatal rat cardiomyocytes [Hilenski et al., 1992]. Thus, FA and costameres seem to play an important role in myofibrillogenesis.

Questions then arise concerning the presence and function of rho A protein in developing cardiomyocytes and whether C3 treatment might destroy the structural integrity of non-striated fibrils, FA, and costameres and further disturb myofibrillogenesis in developing cardiomyocytes. The present study was designed to answer these questions, the aims being (1) to determine if rho A is present in cardiomyocytes by immunostaining with a monoclonal anti-rho A antibody, (2) to examine the effect of C3 on the distribution patterns of vinculin, talin, and integrin in these cells, (3) to analyze the effect of C3 on the process of myofibrillogenesis by examining the distribution patterns of several muscle proteins, and (4) to examine the level of tyrosine phosphorylation in cardiomyocytes following C3 treatment by immunostaining with a monoclonal anti-phosphotyrosine antibody. The results show that rho A is present in the cytoplasm of cultured cardiomyocytes and that its inactivation by C3 treatment results in the disassembly of FA, costameres, and non-striated fibrils.

MATERIALS AND METHODS

Cell Culture

Primary cultures of chicken embryonic cardiomyocytes and fibroblasts were prepared according to Lin et al. [1989]. Ventricular tissues were removed from the hearts of 8-day-old chicken embryos, cut into pieces, and dissociated with 0.05% trypsin-EDTA in Ca^{++}, Mg^{++}-free Hank's buffer. The dispersed cells were plated on collagen-coated coverslips at an optimal culture density of 2–3.5 $\times$ 10^5 cells/ml and maintained in MEM containing 10% fetal calf serum, 2 mg/ml of glutamine, and 100 IU/ml of penicillin, and streptomycin (Gibco, Grand Island, NY) for the first day of culture; this was then changed to glutamine-free medium containing 5 $\times$ 10^{-7} μg/ml of arabinocytosine.

Electroporation

ADP-ribosylation by C3 from *Clostridium botulinum* (Calbiochem-Novabiochem, La Jolla CA) was used to inactivate the small G protein rho A. Before electroporation, day 3 cultures were washed twice with electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, pH 7). The coverslips and the attached cells were transferred in electroporation buffer containing 3 ng/ml of C3 to a Gibco pulser cuvettee, then placed on ice for 5 min. For electropermeabilization, a cell porator (BRL, Gaithersburg, MD) at the settings of 750 v/cm and 60 μF capacitance was used. Controls underwent electroporation in the absence of C3 or in the presence of an unrelated protein such as immunoglobulin (mouse IgG). Incorporated IgG was visualized by subsequent staining with FITC conjugated goat anti-mouse IgG γ chain specific antibody. Immediately after electroporation, the cuvette was placed on ice for another 15 min, then the cells were washed twice with culture medium and maintained at 37°C in an incubator for 1–2 h before staining.

Immunofluorescent Staining

The cell cultures were fixed with 10% formalin in phosphate-buffered saline (PBS) for 10 min and non-specific binding sites were blocked with PBS containing 5% non-fat milk and 0.1% Triton X-100 for 15 min. The cells were washed with PBS, then incubated with primary antibodies at 37°C for 1 h. Mouse monoclonal anti-rho A (26C4) antibody was obtained from Santa Crutz

Biotechnology Inc. (Santa Crutz, CA). Mouse monoclonal anti-vinculin (Sigma, St. Louis, MO), anti-talin (Sigma), and anti-integrinβ1 subunit (Developmental Hybridoma Bank, Y_2E_2, Iowa City, Iowa) antibodies were used at a 1:100 dilution for FA staining. Rat antibodies against α-actinin and M line protein (Wang et al., manuscript in preparation) and mouse monoclonal anti-titin A12 antibody [Wang et al., 1991] were used to study the assembly of myofibrillar proteins in cardiomyocytes. Mouse monoclonal anti-β actin antibody (Sigma) was used to stain nonstriated fibrils in muscle cells. Mouse monoclonal anti-phosphotyrosine antibody (Sigma) was used to study the extent of phosphorylation in cells. An appropriate combination of two primary antibodies was used for double-labeling. Second antibody treatment consisted of incubation of the cells for 1 h, at 37°C with the appropriate antibody chosen from FITC-conjugated goat anti-mouse γ chain-specific antibody, FITC-conjugated goat anti-rat IgG antibody, and biotinylated goat anti-mouse μ chain-specific antibody (combined use with avidin-Texas red) (Vector, Burlingame, CA), followed by rinsing with PBS and mounting in 0.1 M phosphate buffer, pH 8, containing 2% n-propyl gallate and 60% glycerol. Observations were made using a Leica (Buffalo, NY) microscope equipped with epifluorescence illumination.

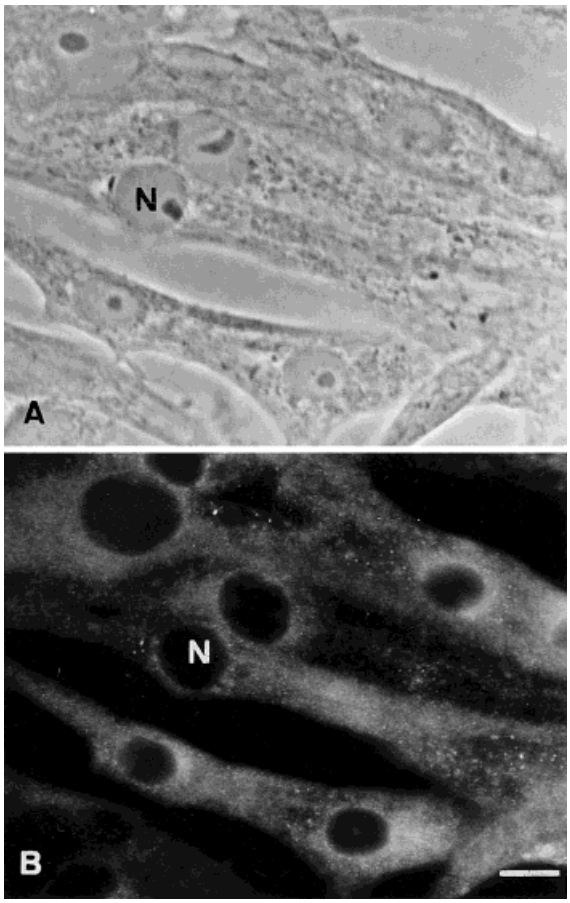

Fig. 1. Distribution of rho A in cultured cardiomyocytes. **A:** Phase image. **B:** Immunostaining with anti-rho A antibody. Most of the cells in the field are cardiomyocytes. Diffuse or punctate staining is seen in the cytoplasm. N, nucleus. Bar = 10 μm.

RESULTS

Rho A Is Present in the Cytoplasm of Chicken Cardiomyocytes

The monoclonal antibody 26C4 was produced against a synthetic peptide (amino acids 120–150 of human rho A) and has been shown by Western blotting to react specifically with rho A in several cell lines [Lang et al., 1993]. In the present study, an amorphous staining pattern was seen in the cytoplasm of cardiomyocytes (Fig. 1). Co-cultured fibroblasts also expressed rho A at a weaker intensity. Since rho A was found to be present in cardiomyocytes, in later experiments, C3 was used to block the function of this protein.

C3 Treatment Induces Disassembly of FA and Costameres

We performed two control experiments; cells were either placed in a blank buffer or buffer containing a control protein (IgG) for electroporation. Neither of both conditions affected the cell morphology. Figure 2 showed an IgG-incorporated cardiomyocyte, which was doubly labelled with anti-titin antibody. The myofibrils in these cells appeared intact (Fig. 2B).

The FA in cardiomyocytes from day 3 cultures were shown by immunofluorescence staining with antibodies against FA proteins, including vinculin, talin, and integrin, to be present as rod- or oval-shaped structures at the ventral attachment sites of myofibrils to the ventral sarcolemma (see Figs. 4 and 5). FA proteins were also found to be localized as rib-like structures on costameres (see Figs. 4B,F and 5B). Vinculin, reported to be localized within the Z-disc in adult cardiomyocytes [Terracio et al., 1990], was only found in FA and costameres (see Fig. 4B). In addition to staining these two

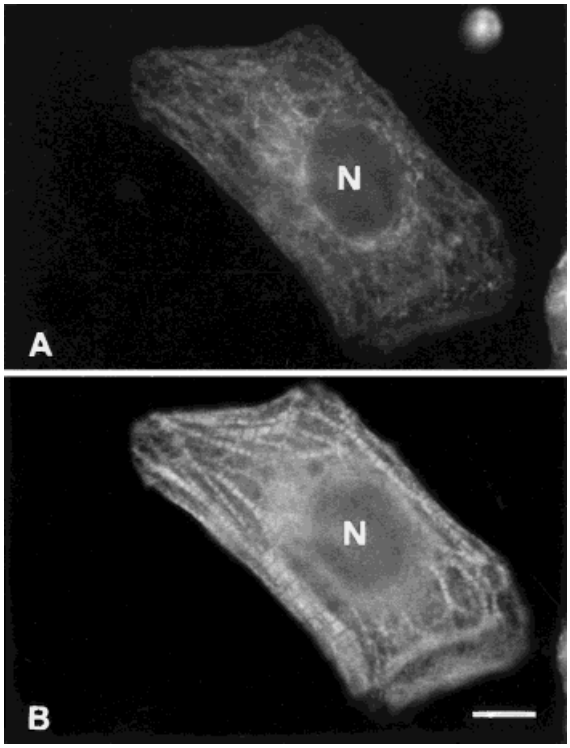

Fig. 2. Incorporation of a control protein in cardiomyocytes by electroporation. Cells are doubly immunostained for incorporated IgG (**A**) and titin (**B**). A normal morphology and sarcomeric architecture is noted. Bar = 10 μm.

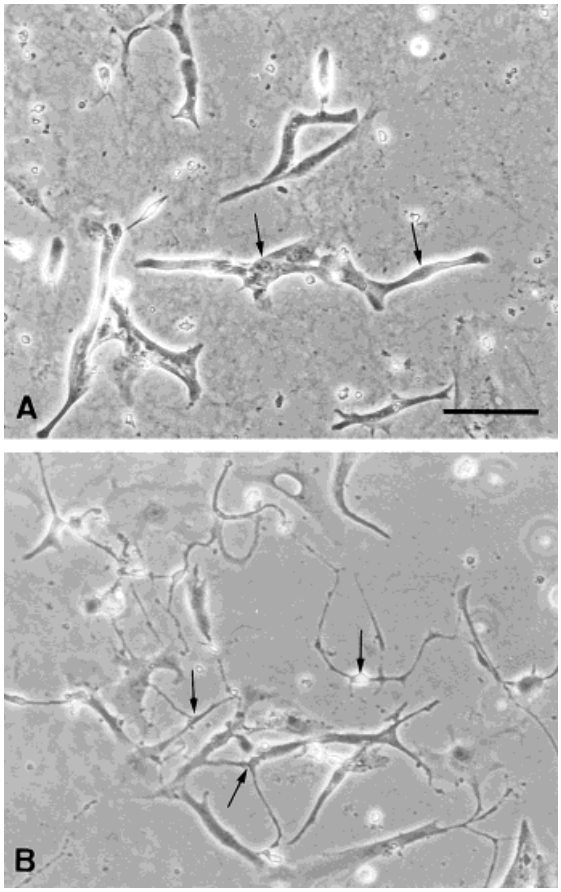

Fig. 3. Effect of C3 electroporation on cardiomyocyte morphology. **A:** Control cells electroporated in blank buffer, showing rod-shaped cardiomyocytes (arrows). **B:** C3 electroporated cells. The cardiomyocyets are smaller and possess thin slender processes. Bar = 100 μm.

structures, anti-integrin antibody also stained some filamentous structures in the ventral sarcolemma (see Fig. 5B). The distribution patterns of FA proteins in normal intact cells and electroporation controls cells were similar.

On the basis of their morphological alterations about 30% of cardiomyocytes were affected by C3 treatment. Following C3 treatment, the cardiomyocytes shrank and displayed several attenuated processes (Fig. 3B), contrasting with the normal elongated or rod-shaped cells (Fig. 3A). Marked changes in the distribution patterns of FA proteins were noted after C3 treatment. Diffuse vinculin and talin immunostaining was seen in the cytoplasm of both cardiomyocytes (Fig. 4D and H) and co-cultured fibroblasts (not shown) and dissolution of FA structures was consistently accompanied by fragmentation of myofibrils (Figs. 4C,G, 5C,E). In addition to this diffuse staining, an unusual aggregated filamentous pattern was also noted in C3 treated cardiomyocytes on staining with anti-integrin antibody (Fig. 5D and F); affected cells showed no costameric staining (Figs. 4D,H, 5D,F).

C3 Treatment Disrupts Assembly of Developing Myofibrils

Cardiomyocytes from day 3 cultures contained many non-striated fibrils, which displayed a continuous staining pattern using anti-β actin (Fig. 6B) or anti-α actinin (Fig. 7A) antibodies, but negative for M-line proteins (Fig. 6A) or titin (Fig. 7B). During development, the non-striated fibrils transformed into immature myofibrils, characterized by segments of sarcomeres interrupted by non-striated segments (Fig. 7A). All myofibrils in mature cardiomyocytes exhibited typical sarcomeric patterns when tested using muscle-specific antibodies. For example, anti-α actinin and anti-M line protein antibodies decorated the Z-line and M-line, respectively. Since the major component in immature cells is non-striated fibril, we were interested in changes in β-actin staining after

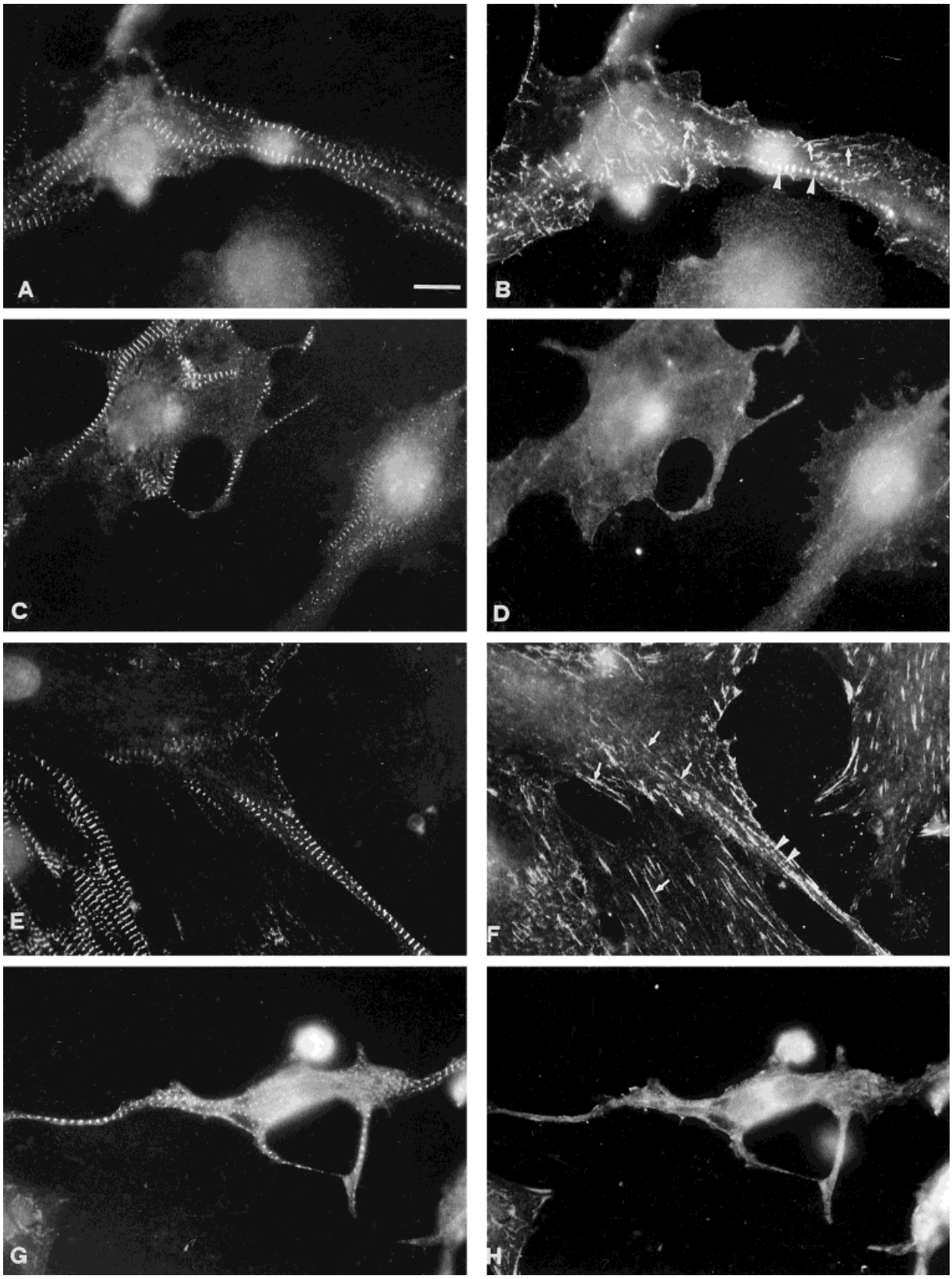

Fig. 4. Effect of C3 treatment on the distribution of vinculin and talin in cardiomyocytes. **A, B** and **E, F:** Control cells. **C, D** and **G, H:** C3-treated cells. Cells were double-labelled with anti-α-actinin (A, C, E, F) and anti-vinculin (B, D) or anti-talin (F, H) antibodies. Focal adhesions shown by anti-vinculin (B, arrows) and anti-talin (F, arrows) antibodies, are oval structures in the ventral surfaces of the cells. Costameres are seen as linear periodic structures (arrowheads in B and F). Both vinculin and talin staining are diffuse (D and H) in C3-treated cells. Bar = 10 μm.

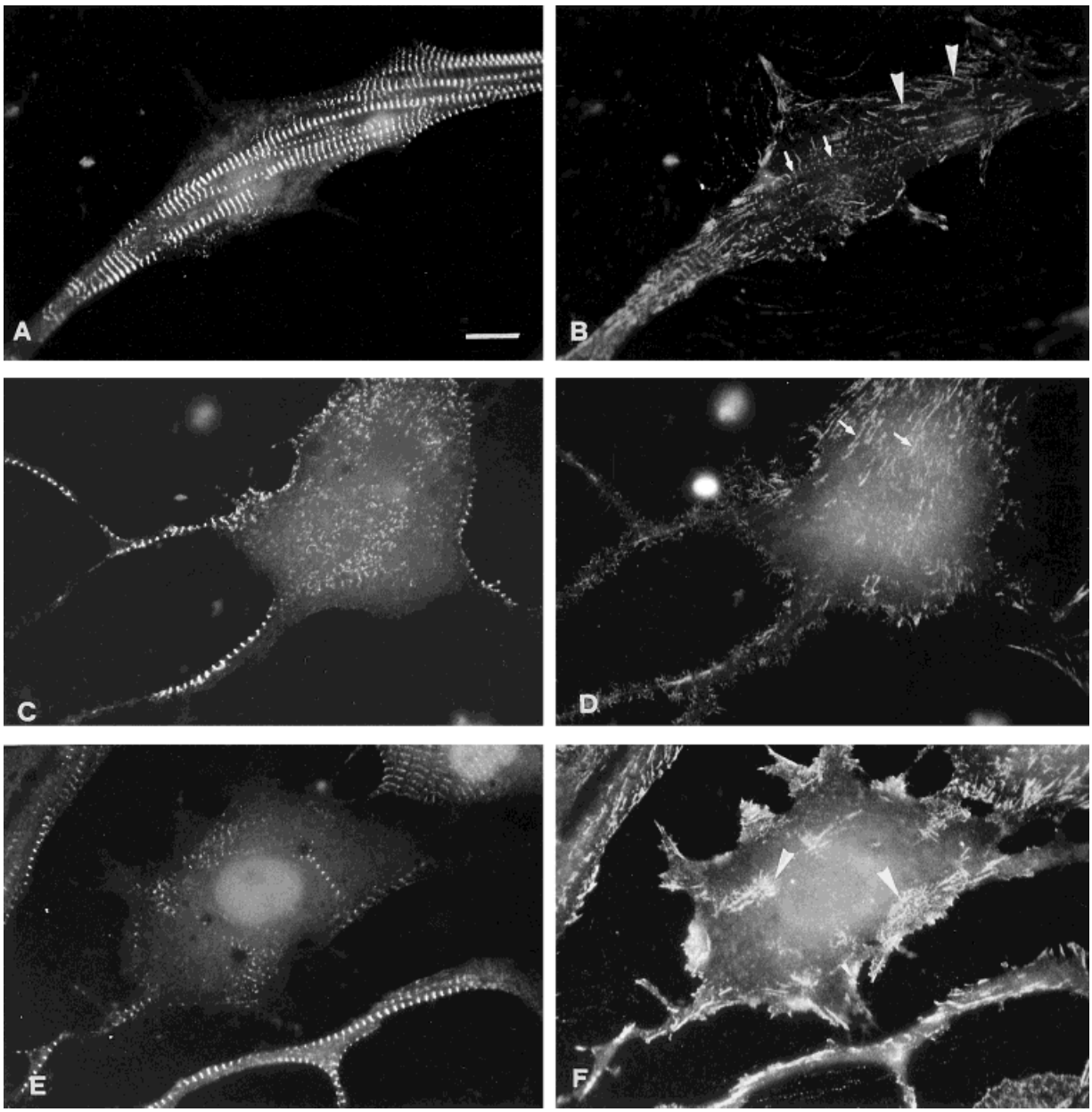

Fig. 5. Effect of C3 treatment on integrin distribution. **A, B:** Control cells. **C–F:** C3-treated cells. Cells were double-labeled with anti-α-actinin (A, C, E) and anti-integrin (B, D, F) antibodies. In control cells, integrin staining is seen on the focal adhesions (arrowheads in B) and costameres (arrows in B); in treated cells, staining is irregular and filamentous (arrows in D) or aggregated (arrowheads in F). Only a few myofibrils are present in C3-treated cells. Bar = 10 μm.

C3 treatment. Depolymerization of non-striated fibrils was frequently seen (Fig. 6D), together with a concomitant decrease in the number of mature myofibrils (Fig. 6C). Figure 7A and B shows the typical staining patterns seen in immature cardiomyocytes using anti-α actinin and anti-titin A12 antibodies. Disruption of myofibrils was seen after C3 treatment (Fig. 7C and D). In some markedly affected cells, α-actinin and titin staining was diffuse (Fig. 7E and F), indicating myofibril disassembly. The effect of C3 on mature cardiomyocytes from older cultures was less obvious; cells became distorted, but myofibrils, though not regularly organized, were still present.

In order to determine the phosphorylation level of FA proteins after C3 treatment, immunofluorescence staining with anti-phosphotyrosine antibody was carried out. Staining was seen on the FA and at the intercellular junction

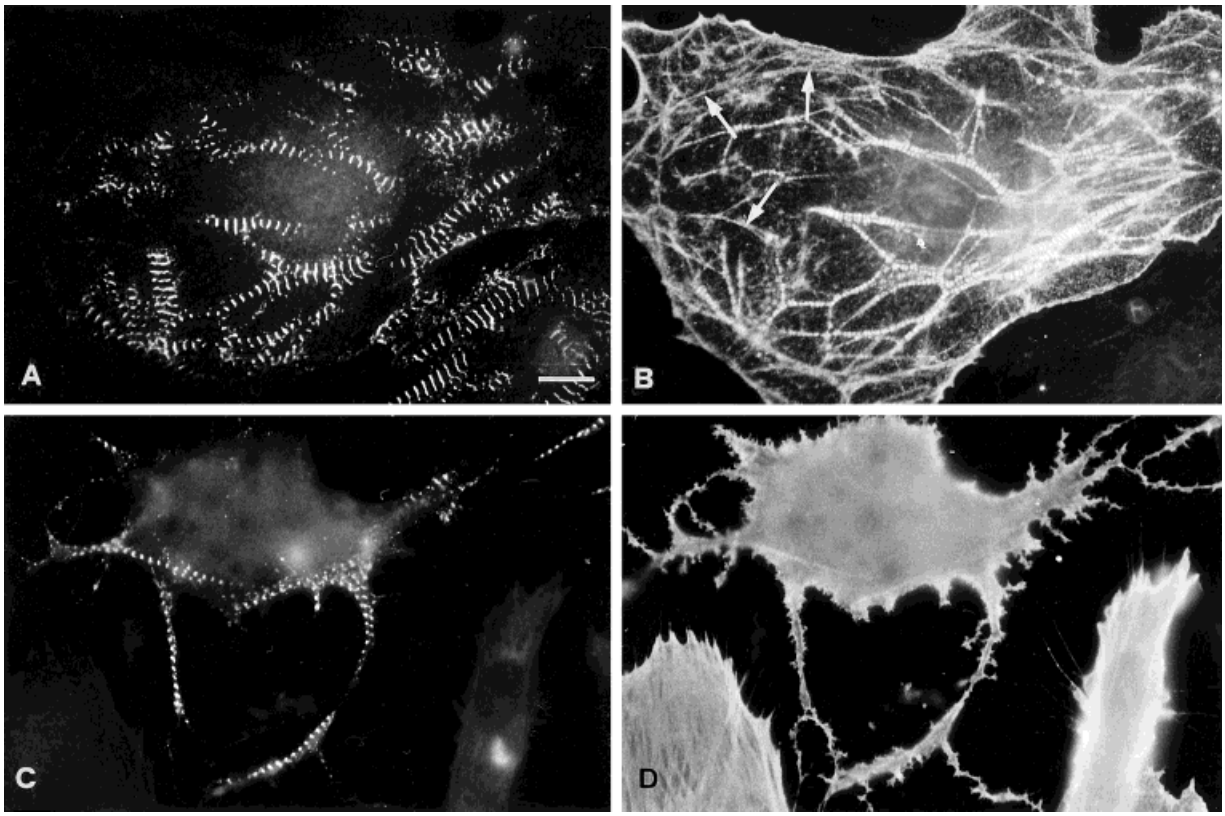

Fig. 6. Effect of C3 treatment on β-actin distribution in immature cardiomyocytes. **A, B:** Control cells. **C, D:** C3-treated cells. Cells were double-labeled with anti-M-line proteins (A, C) and anti-β-actin (B, D) antibodies. In control cells, non-striated myofibrils in immature cells are located peripherally and labeled with anti-β actin antibody (arrows in B), while striated myofibrils are only detected by anti-M-line proteins antibody (A, C). C3 treatment induces depolymerization of non-striated myofibrils (D). Bar = 10 μm.

sites in control cells (Fig. 8B), but diffuse weaker staining was seen throughout the cytoplasm in C3 treated cells (Fig. 8D).

DISCUSSION

Using a monoclonal anti-rho A antibody, we demonstrated that this protein is indeed present in the cytosol of cardiomyocytes and cocultured fibroblasts, which correlates well with the location of rho A in MDCK and Rat-2 cells as reported by Adamson et al. [1992]. The presence of rho A in cardiomyocytes provides the basis for C3 electroporation experiments. Using this approach, a series of morphological changes was seen in C3 treated cardiomyocytes. Since the concentration of C3 used in the present study was quite low (4 μg/ml) and the condition of electroporation was mild in order to prevent cell damage, only 30% of the cardiomyocytes were affected, as judged by their morphological changes.

Several disassembly stages of the FA complex, from random organization to complete depolymerization, were seen in C3 treated cardiomyocytes. In addition, the costameric pattern was lost, as determined by staining with three antibodies directed against vinculin, talin, and integrin. Using double-labelling with muscle-specific protein to examine the status of myofibril assembly, cells containing disrupted FA and costameres were consistently found to exhibit incomplete organization of myofibrils, that is, the myofibrils were either almost absent or were poorly organized as a punctate structure. Since FA and costameres are composed of similar biochemical components (vinculin, talin, and integrin) [McDonald et al., 1995; Pardo et al., 1983], both structures would be expected to be affected by inactivation of rho A. Moreover, both structures are important for cell attachment to the substratum; this may account for the rounding-up of cells seen after C3

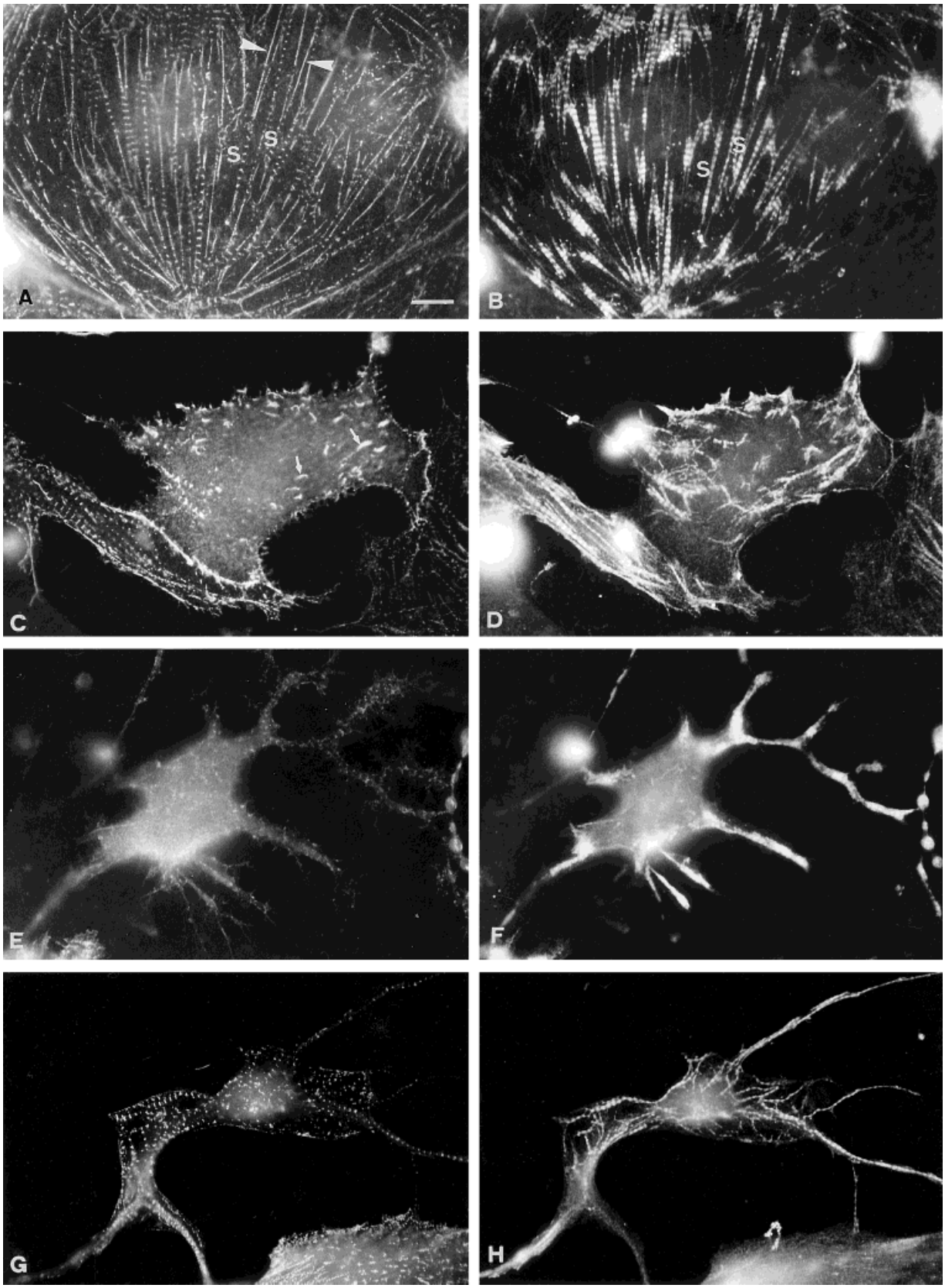

Fig. 7. Distribution of α-actinin and titin in C3-treated immature cardiomyocytes. **A, B:** Control cells. **C–H:** C3-treated cells. Cells were double-labeled with anti-α-actinin (A, C, E, G) and anti-titin (B, D, F, H) antibodies. Non-striated myofibrils are stained in a continuous pattern by anti-α actinin antibody (arrowheads in A), but not by anti-titin (B) antibody. Striated myofibrils (S) are stained with both anti-α actinin and anti-titin antibodies. In C3-treated cells, myofibrils are fragmented (arrows in C). A less affected cell (lower left) shows a typical sarcomeric staining pattern with anti-α actinin and anti-titin antibodies (C, D), while in more severely affected cells, filamentous staining is seen for both α-actinin (E) and titin (F). Some C3-treated cells appear twisted, although their myofibrils are intact (G, H). Bar = 10 μm.

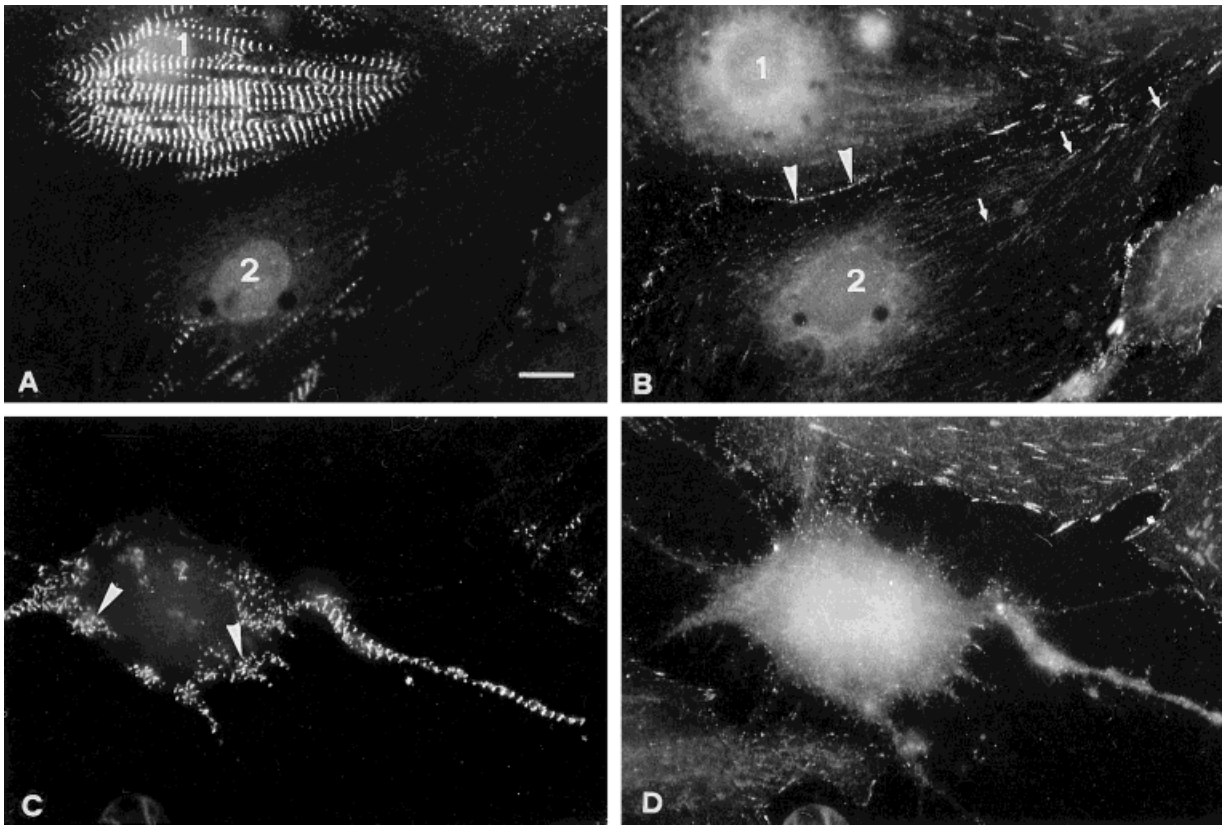

Fig. 8. Effect of C3 treatment on the distribution of phosphotyrosinated proteins in cardiomyocytes. **A, B:** Control cells. **C, D:** C3-treated cells. Cells were double-labeled with anti-M-line protein (A, C) and anti-phosphotyrosine (B, D) antibodies. Anti-phosphotyrosine antibody stains both focal adhesion components (arrows in B) and cellular junction components (arrowheads in B) in control cardiomyocytes; in C3-treated cells, staining for phosphotyrosine is diffuse (D). The myofibrils in this cell are fragmented as small clumps (arrowheads in C). Bar = 10 μm.

treatment in the present study. The activity of integrin has been shown to be crucial in initiating new myofibril formation in the developing muscle cells. For example, addition of anti-integrin β1 antibody to chicken myotube [McDonald et al., 1995] or rat cardiomyocyte cultures [Hilenski et al., 1992] inhibits sarcomere assembly. In Drosophila, myogenesis and Z-band formation are defective in myospheroid mutants defective in the β-integrin gene [Volk et al., 1990]. Our results also confirm that intact FA and costameres are essential for myofibrillogenesis in chicken cardiomyocytes. Although the FA acts as a nucleating center for developing myofibrils [Lu et al., 1992], no functional study has yet been reported and the C3-induced FA depolymerization serves as a useful model for studying the role of FA in myofibrillogenesis.

Concomitant with FA and costamere breakdown, staining of C3 treated cells for phosphotyrosine revealed a diffuse pattern in the cytoplasm instead of the spot-like pattern seen on the FA and intercellular junctions of control cells. Since tyrosine phosphorylation of paxillin and $pp125^{FAK}$ occurs upon activation of rho A and is necessary for new assembly of FA and stress fiber formation Swiss 3T3 fibroblasts [Burridge et al., 1992; Barry and Critchley, 1994; Ridley and Hall, 1994; Flinn and Ridley, 1996], the mechanism of disassembly of FA in cardiomyocytes by C3-induced inactivation of rho A may be due to dephosphorylation of FA components. Recent study of Chranowska-Wodnicka and Burridge [1996] has shown that cell contractility stimulated by activation of rho induces the formation of focal adhesion and stress fibers in 3T3 fibroblasts. Further study is necessary to determine whether C3 treatment inhibits the contractility of cardiomyocytes prior to the disassembly of focal adhesion.

Immature myofibrils are more sensitive than mature myofibrils to C3 treatment. Since mature myofibrils consist of the muscle isoforms of

actin, tropomyosin, myosin, and α-actinin [Wang et al., 1988, 1990; Schulthesis et al., 1990], they are probably more resistant to C3 treatment either via a different interaction with FA or rho A. Immature myofibrils are composed of either striated and non-striated segments or entirely of non-striated segments. Non-striated segments are composed of non-muscle isoforms of actin, tropomyosin, myosin, and α-actinin [Lin and Lin, 1986; Schulthesis et al., 1990; Wang et al., 1990; Handel et al., 1991], a protein profile similar to that seen in stress fibers in non-muscle cells [Bretscher, 1991]. Following C3 treatment, β-actin-positive, non-striated segments became fragmented and even depolymerized. This result correlates well with the dissolution of stress fibers seen on microinjection of C3 into Swiss3T3 fibroblasts [Paterson et al., 1990; Ridley and Hall, 1994]. Once non-striated segments were broken down, striated segments of immature myofibrils soon collapsed. Since non-striated segments develop into mature myofibrils, the resultant shortage of myofibril presursors would lead to a decrease in mature myofibrils in C3 treated cardiomyocytes. Thus, a plausible hypothesis would be that C3 treatment perturbs myofibrillogenesis via a mechanism involving the destruction of FA, costameres, and non-striated fibrils.

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