Protein Kinase C-Mediated Desmin Phosphorylation Is Related to Myofibril Disarray in Cardiomyopathic Hamster Heart[1]

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The cardiomyopathic (CM) Syrian golden hamster (strain UM-X7.1) exhibits a hereditary cardiomyopathy, which causes premature death resulting from congestive heart failure. The CM animals show extensive cardiac myofibril disarray and myocardial calcium overload. The present study has been undertaken to examine the role of desmin phosphorylation in myofibril disarray observed in CM hearts. The data from skinned myofibril protein phosphorylation assays have shown that desmin can be phosphorylated by protein kinase C (PKC). There is no significant difference in the content of desmin between CM and control hamster hearts. However, the desmin from CM hearts has a higher phosphorylation level than that of the normal hearts. Furthermore, we have examined the distribution of desmin and myofibril organization with immunofluorescent microscopy and immunogold electron microscopy in cultured cardiac myocytes after treatment with the PKC-activating phorbol ester, 12-*O*-tetradecanylphorbol-13-acetate (TPA). When the cultured normal hamster cardiac cells are treated with TPA, desmin filaments are disassembled and the myofibrils become disarrayed. The myofibril disarray closely mimics that observed in untreated CM cultures. These results suggest that disassembly of desmin filaments, which could be caused by PKC-mediated phosphorylation, may be a factor in myofibril disarray in cardiomyopathic cells and that the intermediate filament protein, desmin, plays an important role in maintaining myofibril alignment in cardiac cells. Exp Biol Med 227:1039–1046, 2002.

Key words: cardiac hypertrophy; myocardium; intermediate filaments; myofibril protein; cell culture

Intermediate filaments (IF) represent a unique group of cytoskeletal structures present in the cytoplasm of muscle cells. A number of IF proteins have been recognized in muscle cells: desmin, vimentin, nestin, synemin, paranemin, lamins, and cytokeratins. However, the main muscle-specific IF protein is desmin (1). In cardiac muscle, desmin is increased at intercalated discs, the attachment between cardiomyocytes. Desmin is the first muscle-specific protein to appear during myogenesis (1). Desmin-related myopathies are marked by accumulation of desmin, which is often familial and associated with cardiomyopathy (CM) (2–4). Moreover, an abnormal accumulation of phosphorylated desmin filaments was observed in skeletal muscle of a human familial CM (5).

The hereditary CM, an autosomal recessive disease, found in Syrian hamsters (strain UM-X7.1) offers an important model of myocardial disease resulting in chronic congestive heart failure (6). It is characterized by the early development of focal myolytic and necrotic lesions, randomly distributed throughout the myocardium followed by ventricular dilatation and hypertrophy. Several sites of cellular dysfunction have been identified in the CM myocardium. These include defects in the mitochondria, sarcoplasmic reticulum, myofibrils, and sarcolemma (7). Such studies are important in that they have indicated a similarity between the pathology of the hamster CM and that of human heart disease. For example, the disorientation of myofibrils is a rather classical trait of hypertrophic obstructive CM in humans (8, 9). Although many reports exist on the morphological, physiological, or biochemical abnormalities of this disease, a primary cellular defect leading to CM has yet to be characterized.

Recently, it has been reported that protein kinase C (PKC) activity and contents were significantly increased in the CM hamster hearts (Strain UM-X7.1) (10). In addition, increased PKC activity and PKC expression have been observed in the failing heart of the patients with CM (11). To

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further understand the function of the desmin in myofibrillogenesis and in the heart defect of the CM, we have compared the distributions and phosphorylation properties of desmin both in cultured hamster cardiac cells and in *in vivo* CM hamster hearts. Our results have demonstrated for the first time that desmin in skinned myofibrils can be phosphorylated by PKC. This is consistent with the report that purified desmin can be phosphorylated by PKC (12). The desmin from CM hearts, which show significant myofibril disarrays, has a higher phosphorylation level than that of normal hearts. When cultured normal cardiac cells are treated with PKC activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), desmin filaments are disassembled and the myofibrils become disarrayed. These results suggest that disassembly of desmin filaments may be a factor in myofibril disarray in CM cells and that the intermediate filament protein, desmin, plays an important role in the maintenance of alignment in cardiac myofibrils.

Materials and Methods

Hamster System. Genetically CM, Strain UM-X7.1, and control Syrian hamsters were obtained from our colony maintained in the Central Animal Care Facility at the State University of New York, Health Science Center at Syracuse. Normal and CM animals were housed under identical conditions in the same room at 23°C on a light cycle of 12:12-hr light:dark. They were fed Purina Lab Chow and water *ad libitum* with a supplement of hamster seed mixture and lettuce. Hearts from animals at 6 and 8 months of age were used in the electrophoresis and frozen section immunofluorescent studies. Hearts from newborn animals were used in tissue culture.

Cardiac Tissues. *Immunohistochemical Studies.* The hearts of 6-month-old normal and CM hamsters were extirpated after anesthetizing the animals in a metofane atmosphere followed by cervical dislocation. Whole hearts were rinsed briefly in ice-cold phosphate-buffered saline (PBS) to remove blood and tissue debris. The atria and major vessels were trimmed away and discarded, leaving only the ventricular portion of the heart. The ventricles were cut into 1-mm^3 pieces and mounted onto steel stubs using 20% sucrose in 0.10 *M* phosphate buffer, pH 7.4. The tissues were mounted on stubs, rapidly frozen in liquid nitrogen, and stored at −70°C. Sections of 1–2 μm thickness were cut using glass knives, thaw-mounted onto albumin-coated slides, and dried at room temperature prior to immunofluorescent staining. The sections were then minced in PBS-0.1% sodium azide and were preincubated in PBS-3% nonfat milk for 15 min to block nonspecific staining. The primary antibodies, monoclonal anti-desmin from Sigma Chemical (St. Louis, MO), diluted to 1:20 with PBS, were placed over the sections and left for 60 min in a humid chamber at room temperature. The samples were washed three times in PBS-0.1% sodium azide for 5 min each, and placed again in PBS-3% milk. Fluorescein-conjugated secondary antibodies diluted to 1:20 with PBS were then placed over the sections for 60 min at room temperature. The preparations were washed three times for 5 min each in PBS, mounted in N-propyl gallate and glycerol, and viewed with a Universal Light microscope equipped with epifluorescent illumination using a mercury vapor light source (Zeiss, Jena, Germany).

Immunoprecipitation. Cardiac cells from normal and CM hamsters were lysed in cold lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium desoxycholate, 150 m*M* NaCl, 1 m*M* EGTA, and 50 m*M* Tris-HCl, pH 7.5). Cell extracts were homogenized by sonication and repeated pipetting. Two hundred micrograms of myofibril isolate was incubated with 2 μg of polyclonal antidesmin for 1 hr at 4°C. Isolates were then treated with Protein A Sepharose according to the manufacture's guidance (Amersham, Piscataway, NJ). The pellet was washed three times in ice-cold buffer, and was loaded for SDS-PAGE analysis.

Immunoblotting. For Western blot assays, the proteins from SDS-PAGE were transferred onto nitrocellulose membranes followed by incubation with specific antibodies (13). A 3% milk solution in immunoblot buffer (150 m*M* NaCl and 50 m*M* Tris, pH 7.8) was used to block specific reactions prior to overnight incubation in antidesmin antibody diluted 1:1000 with immunoblot buffer. A secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) was diluted 1:3000 with immunoblot buffer and was incubated with the blot for 1 hr at room temperature. The Western blotting method was also used to quantify the phosphorylated serine levels in immunoprecipitated desmin from the normal and CM hamster hearts. Monoclonal antiphosphoserine (Sigma) was used at a dilution of 1:25, and the secondary antibody concentration was 1:500. The antibody binding was detected by an enhanced chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL). Three to five animal hearts were used for each experiment. Protein loads were standardized by a bicinchoninic acid protein assay before electrophoresis and by quantitative densitometry of Coomassie Blue-stained gels. Blots were stained with 0.1% Ponceau S solution to visualize protein bands and to confirm both consistent protein loading among wells and complete transfer of proteins to blots. ECL bands were scanned and analyzed with an AlphaImager Digital Imaging System and AlphaEase software (Alpha Innotech, San Leandro, CA).

Phosphorylation Assays. Phosphorylation samples of the heart tissues were prepared from 6-month-old normal and CM hamsters by the methods described previously (14). Briefly, the ventricular tissues were diced and homogenized for 3 to 5 sec in 5 ml of relaxing solution using a Polytron homogenizer. Cells were collected by differential centrifugation on a tabletop centrifuge and were then incubated for 6 min at room temperature in relaxing solution containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin (BSA). First, we did Western blot assays to confirm that desmin was still connected with these skinned myofibrils. These skinned myofibrils were then used as substrate to

detect PKC- mediated phosphorylation. Myofibril proteins (0.2 mg) were incubated at 30°C for 30 min with 1 unit of PKC plus PKC activator, 100 n*M* TPA and 50 μ*M* phosphatidylserine in the presence of ^{32}P-ATP. Myofibril protein phosphorylation was detected by separating the protein samples on SDS-PAGE. Gels were stained with Coomassie Blue, dried, and subjected to autoradiography.

Cultured Cardiac Cells. *Tissue Culture of Cardiac Myocytes.* Myocytes were isolated from heart ventricles of 3-day-old normal and CM hamsters. Between 20 and 50 animals from each strain were used in each experiment. The animals were sacrificed by cervical dislocation and the hearts were immediately removed using sterile techniques. The extirpated hearts were washed three times in cold Hanks' solution (Gibco-BRL, Grand Island, NY) to remove residual blood. The ventricles were dissected free and minced into very small pieces using a new scalpel under a dissecting scope. The pieces were washed twice with ice-cold Hanks' solution, and were then treated with 0.08% trypsin and 0.01% collagenase in Hanks' solution for 10 min at 37°C in an incubator with gentle agitation. The first supernatant was discarded and the three additional supernatants were diluted 2-fold with cold culture medium containing Earle's minimum essential medium, 15% fetal calf serum, 200 m*M* glutamine, 100 μ/ml penicillin, and 100 mg/ml streptomycin. Cells were harvested from the enzymatic solution by centrifugation at 100*g* for 2 min and fresh culture medium was added to resuspend the cells. To enrich for myocytes, a differential adhesion step was used. To accomplish this, the dissociated heart cell suspensions were preincubated in a culture dish for 60 min at 37°C. Most of the fibroblasts attached to the bottom of the dish during this period. The remaining unattached cells (containing mostly myocytes) were diluted to a final density of 2×10^5 dispersed cells/ml medium. The cells were grown on gelatin-coated glass microscope coverslips, placed in 35-mm diameter plastic tissue culture dishes for immunofluorescent microscopic study, or grown on 100-mm diameter plastic tissue culture dishes for electrophoresis study. The cells were incubated at 37°C in a 5% carbon dioxide and 95% air mixture. The culture medium was changed every other day.

Treatment of cells with TPA. TPA was obtained from Sigma. Stock TPA solution was prepared by dissolving the agent in absolute ethanol (1 mg/ml). TPA stock was stored at –20°C, and fresh TPA medium was prepared daily and added directly to the growth medium of the cells in culture to give a final concentration of 50 ng/ml culture medium. The same amount of ethanol diluted by culture medium without TPA was added to the control cultures. Treated cells were incubated at 37°C for 10, 30, or 60 min of exposure to TPA. After incubation, the cells were washed twice with fresh culture medium followed by fixation and staining for indirect immunofluorescent and immunoelectron microscopic studies.

Indirect Immunofluorescence. Immunofluorescent studies were performed on cultured cardiac myocytes to localize desmin. After the cultured cells were minced in PBS-0.1% sodium azide, they were preincubated with PBS-3% nonfat milk for 15 min to block nonspecific staining. The monoclonal antidesmin antibodies were placed over the cells and incubated for 60 min in a humid chamber at room temperature for 1 hr. The samples were washed three times in PBS-0.1% sodium azide for 5 min each, and again placed in PBS-3% milk. Fluorescein-conjugated secondary antibodies were placed over the cells for 60 min at room temperature. The preparations were washed in PBS, 3 × 5 min each, mounted in *N*-propyl galate and glycerol, and viewed with a Universal Light microscope equipped with epifluorescent illumination (Zeiss).

Data Analysis. Protein phosphorylation and Western blotting were quantitatively measured by densitometry (AlphaImager Digital Imaging System and AlphaEase software). Data were expressed as mean ± SE. Statistical analysis was carried out using analysis of variance (ANOVA) and Student's *t* test for unpaired observations. Significance was defined at the level of $P < 0.05$.

Results

Cardiac Tissues. *Electron Microscopic (EM) and Immunohistochemical Analyses.* To define the desmin distribution in cardiac myocytes, EM and immunohistochemical studies were performed on both normal and CM hamster hearts. Myofibril disarray is obvious in CM hamster heart samples when examined under the electron microscope (Fig. 1B). Normal heart samples contain well-organized myofibrils that are in parallel arrangements with respect to each other (Fig. 1A). These observations corroborate the immunofluorescent data. Unfixed frozen sections of ventricular myocardium from normal and CM hamsters show different staining patterns using monoclonal antibodies to desmin. In normal cardiac tissue, desmin staining is localized at the intercalated discs and in the intermyofibrillar material located between the Z-lines. The sections of normal cells labeled with antidesmin antibody show that the organized myofibrils, intercalated discs, and Z lines stain intensely (Fig. 1C). In sections of CM hamster heart tissue labeled with monoclonal antidesmin, the Z lines and intercalated discs stain diffusely as compared with the normal ones. Moreover, myofibril disarrays in CM heart tissues are rather obvious (Fig. 1D).

Western Blotting and Protein Phosphorylation Assays. We observed the changes in desmin distribution and myofibril disarray in cardiac tissues from CM hamsters. We tried to demonstrate whether there was a quantitative change of desmin in CM hearts compared with the normal ones. Western blotting assays were performed, using monoclonal antibody antidesmin, to quantitatively detect the desmin content in cardiac tissues from CM and control hamster hearts. The results showed that a band at the 55 kDa level is detected in both samples, and that there is no significant difference in desmin content between CM and normal control hamster hearts ($P > 0.05$; Fig. 2). The data also dem-

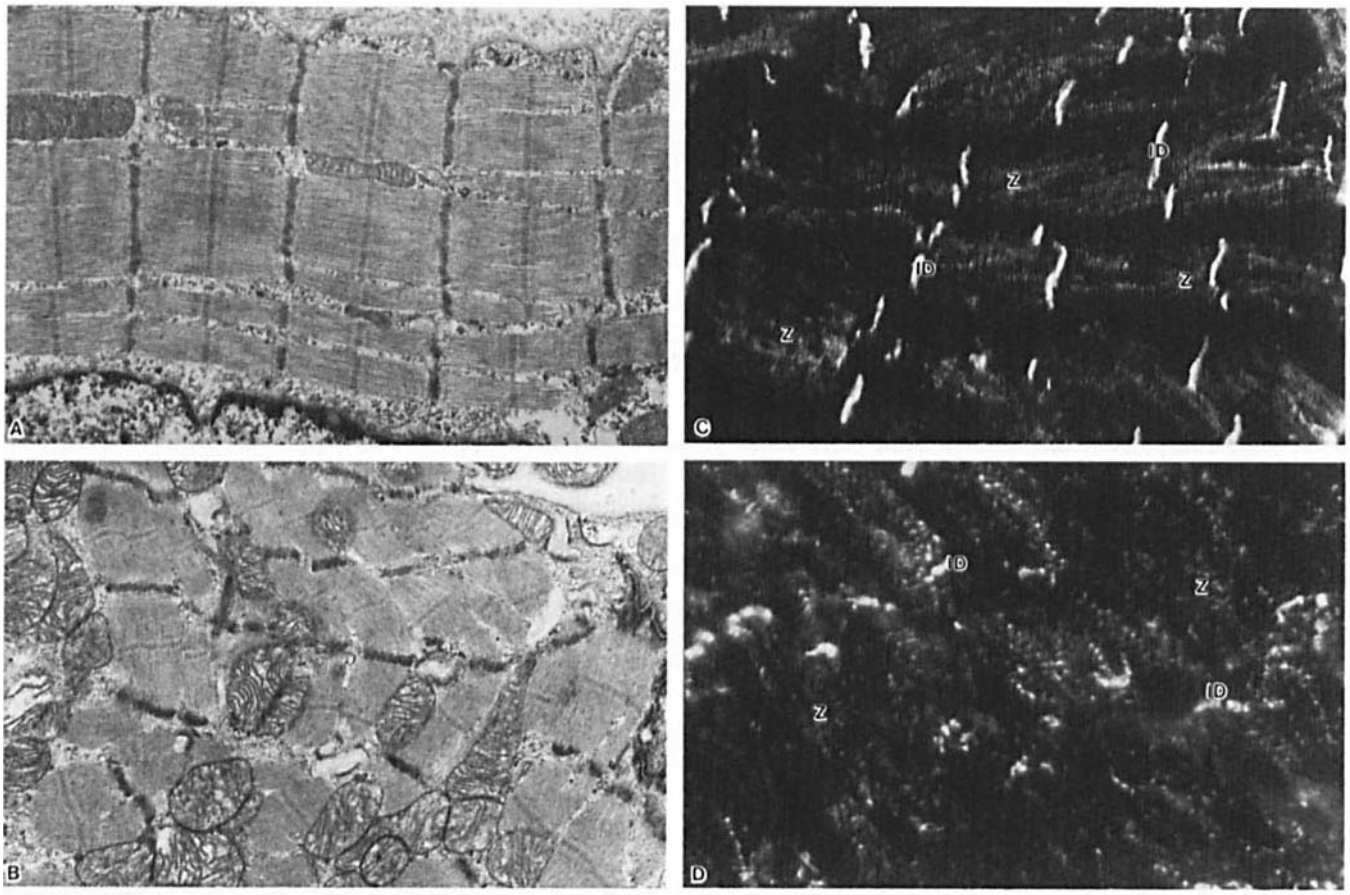

Figure 1. Electron microscope and immunofluorescent analyses of hamster heart samples. Myofibril disarray is obvious in cardiac section from CM hamster heart (B) compared with the normal control hearts (A). Unfixed, frozen sections of cardiac muscle are stained with monoclonal antidesmin antibody. Note the staining at the intercalated discs and Z-lines in the myofibrils. The localization of desmin in CM hamster heart tissue (D) appears more diffuse than in normal tissue (C). ID, intercalated disc; Z, Z-line.

onstrate that desmin is still connected with myofibrils in skinned myocytes. Because desmin was reported as a phosphoprotein, we further investigated the phosphorylation status of desmin by PKC in CM and control hamster hearts. At first, we detected the phosphorylation status of desmin in intact myofibrils. After a 30-min incubation of myofibrils with PKC, we found that PKC specifically phosphorylated troponin I (34 kDa). PKC caused the phosphorylation of a protein at the 55 kDa level, where desmin is located (Fig. 3). The results indicate that by using myofibrils as substrate, PKC is able to phosphorylate desmin. This is consistent with the report that purified desmin protein can be phosphorylated by PKC *in vitro* (12). To further detect the phosphorylation status of desmin in CM and control hamster hearts, we precipitated desmin molecules from CM and normal control hamster hearts and evaluated the phosphoserine contents in the same amounts of desmin from either the CM or normal control hearts. The results clearly demonstrate that the desmin from CM hearts contains more phosphoserine than that of normal control hearts ($P < 0.01$; Fig. 4). These results suggest that in CM cardiac myocytes, desmin is overphosphorylated.

Cultured Cardiac Cells. In an attempt to determine whether the desmin overphosphorylation causes the myofibril disarray and heart failure in CM hamsters or is a secondary effect of the heart failure in CM hamsters, we did experiments to analyze the effects of PKC-mediated phosphorylation in cultured hamster cardiac cells and we investigated the consequences of this phosphorylation.

Immunofluorescent Analysis. Indirect immunofluorescent staining with monoclonal antidesmin was used to analyze the degree of alteration of the desmin filaments in cultured normal hamster myocardial cells after treatment with TPA. As assessed by phase and indirect immunofluorescent microscopy, treatment with TPA for 30 min produced significant changes in the distribution of desmin and the organization of myofibrils in normal myocardial cells, which were very similar to that observed in CM hamster heart cells in culture. In control cells, the desmin proteins were localized in the Z lines, which exhibit a striated pattern in the normal cardiomyocytes after 5 days in culture (Fig. 5A). Phase contrast optics shows that myofibrils organize relative to each other in the same cell. In contrast to the control cells, the cells treated with TPA for 30 min no longer show desmin associated with the Z lines; instead, desmin is localized in the cytosolic areas (Fig. 5B). Alteration of desmin distribution in normal myocytes shows similar results after incubation of TPA for 10 and 60 min

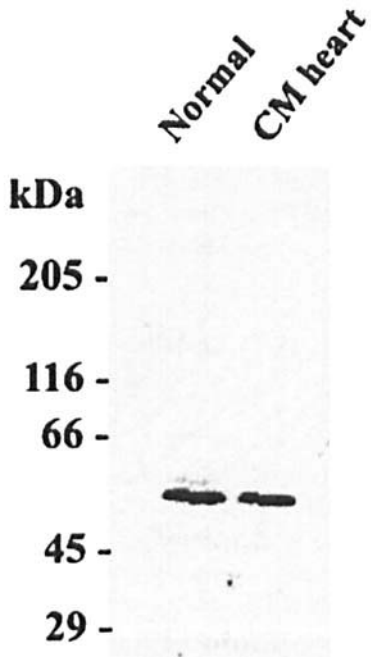

Figure 2. Western blotting analysis of desmin in normal and CM hamster heart tissues. After the separation of the equal amount of cardiac proteins from normal and CM hearts with SDS-PAGE, a protein with an approximate molecular weight of 55 kDa was decorated by a monoclonal antibody antidesmin. Densitometry results from blots of three separate experiments show that the integrated density values (mean ± SE) of the 55-kDa band for normal and CM heart are 39,521 ± 8,775 and 31,785 ± 6,903, respectively. $P > 0.05$.

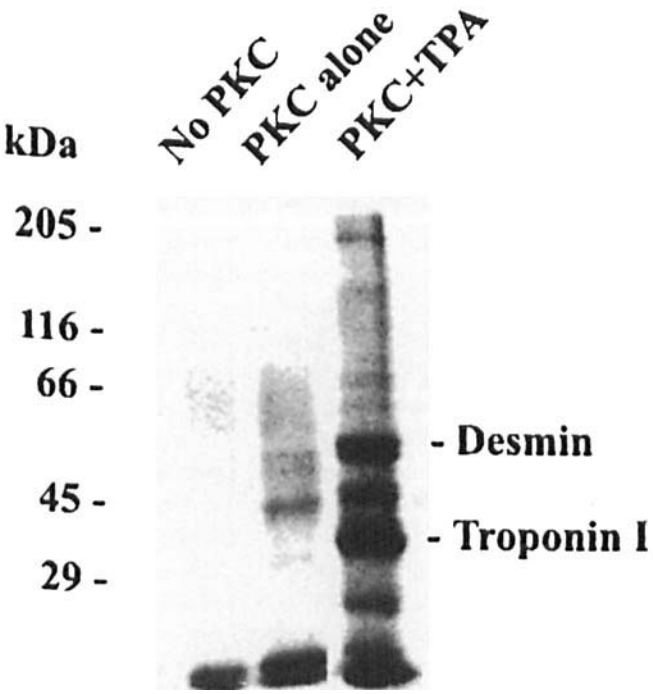

Figure 3. Phosphorylation of myofibril proteins form hamster hearts. In skinned hamster cardiac myocytes, desmin and troponin I are two major proteins phosphorylated by PKC.

(not shown). This change of desmin distribution after TPA treatment is similar to the desmin staining pattern observed in CM myocytes at the same age in culture (Fig. 5C). In

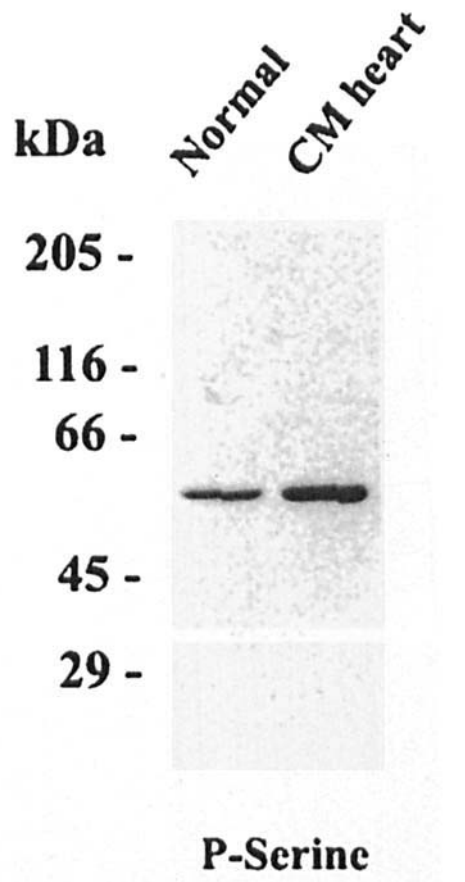

Figure 4. Antiserine staining of the precipitated desmin molecules from CM and normal hamster hearts (N). SDS-polyacrylamide gel shows the immunoprecipitate (~55 kDa) by antidesmin antibody from the normal and CM hamster hearts. Phosphorylated serine (P-Serine) in the protein is revealed by a monoclonal anti-phosphoserine. Densitometry results from blots of four separate experiments show that the integrated density values (mean ± SE) of the band for normal and CM heart are 34,502 ± 5,533 and 58,911 ± 9,350, respectively. $P < 0.01$.

addition, myofibril disarray can be observed by phase contrast optics in Figure 5. Thus, the TPA treatment results in changes to the normal cells that closely mimic the CM heart cell phenotype, suggesting that PKC pathway may very well be involved in the myofibril disarray and hypertrophy in CM hamster hearts.

Discussion

Desmin is a major intermediate filament protein found exclusively in striated (skeletal or cardiac) muscle tissues. It plays an essential role in the maintenance of muscle cytoarchitecture by anchoring neighboring Z discs together (15, 16). Two types of distribution patterns for desmin have been described in cardiac muscle cells *in vitro* from embryonic and neonatal rat. One is demonstrated as a filamentous network of desmin filaments localized in a regular periodic pattern in the regions of the Z lines and intercalated disks (17). Another shows a component of cytoplasmic filamentous structures, which comprise a network distinct from actin filament bundles and microtubules in chicken skeletal muscle cells (18). Research on freshly isolated rod-shaped

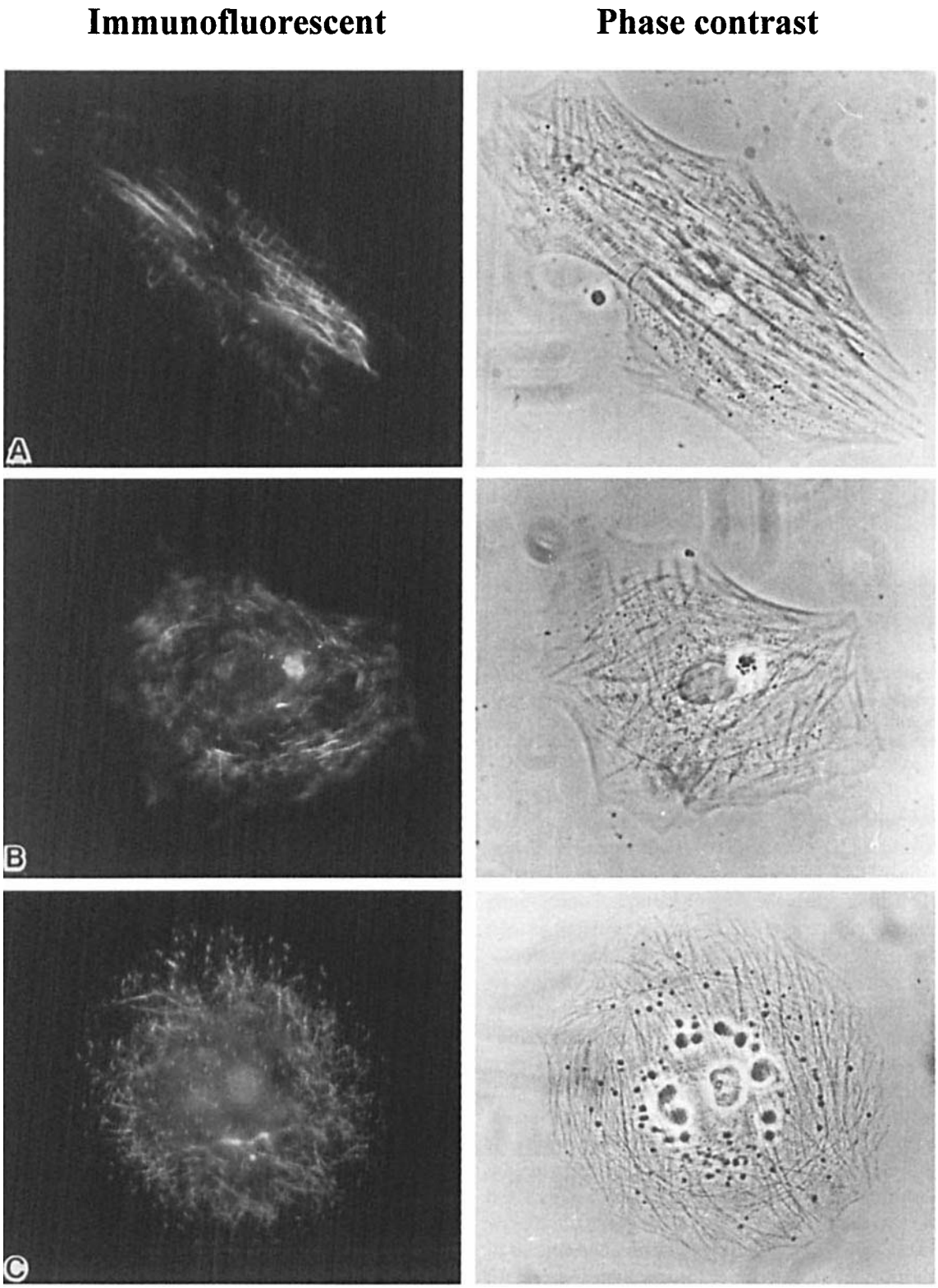

Figure 5. Immunofluorescent and matching phase contrast micrographs of cultured cardiac myocytes from normal and CM hamsters stained with antidesmin monoclonal antibody. (A) Desmin is localized in the Z band areas and forms a striated pattern in normal cultured cells. Myofibrils are in parallel alignments and do not show disarray. (B) Desmin is spread into the cytosolic areas and the filaments to disassemble in the normal cultured cells treated with TPA. The phase contrast micrograph shows myofibril disarray in this normal cell treated with TPA. (C) Desmin does not form the typical striated pattern in CM cardiac cells stained with antidesmin antibody. The phase contrast micrograph shows myofibril disarray in the CM heart cell.

cardiac myocytes from rat show that desmin labeling is confined to the intermyofibrillar spaces (19); this corroborates the immunoelectron microscopic studies on desmin in chicken ventricular papillary muscle and in cultured cardiac myocytes from hamster (20). At the electron microscopic level, the intermediate filaments labeled with polyclonal antidesmin, in combination with an indirect immunoferritin approach, exhibit a highly curvilinear and undulating pattern in most regions of the cell, forming an intricate roof around all of the myofibrils (21). Our immunofluorescent studies show a typical pattern of Z line and intercalated disc localization for desmin in normal hamster hearts; however, CM hearts show a much more diffuse pattern of staining for desmin. The difference in immunofluorescent staining for desmin in normal and CM hamster hearts suggests a relationship between the properties of the protein, its biochemical characteristics, and its physiological function(s).

A temporal relationship between changes in intermediate filament organizations and alteration in phosphorylation of their subunit proteins has been demonstrated. These observations led to speculation that phosphorylation plays an important role in regulating organization of the intermediate filament component of the cytoskeleton (12). Desmin is a phosphoprotein with phosphorylation sites of multiple serine residues. Analysis of specific fragments of desmin indicates that the purified desmin protein can be phosphorylated by PKC (22). Kitamura *et al.* (12) reported several serine residues in the desmin molecule, Ser-12, Ser-29, Ser-38, and Ser-56, could be phosphorylated by PKC. In our studies, we have found that desmin is still connected with myofibrils in skinned myocytes and desmin in myofibrils is easily phosphorylated by PKC. It has also been found that desmin loses its ability to form intermediate filaments when phosphorylated *in vitro* by PKC (23). These researchers also showed that the desmin phosphorylated by PKC does not polymerize; moreover, polymerized filaments tend to depolymerize after phosphorylation (23).

Several papers have been published regarding the abnormal accumulation of desmin in human CM (3, 24). In some cases, CM show homogenous degeneration of the central portions of the involved muscle fibers. These degenerative sites are characteristically stained by antibody against desmin (25). Previously reported biochemical studies on skeletal muscle in a familial muscle disorder have revealed a large increase in the amount of desmin in phosphorylated forms (5).

Myofibril disarray, as a morphological characteristic, is found in both intact cardiac tissue and cultured cells from CM hamster (8, 9, 26). The relationship between accumulation of desmin and myofibril disarray in these CM is not clear. In our studies, we have not found any significant difference in desmin concentration between cardiac tissues from CM or from normal control hamsters. However, we have discovered that the phosphorylation level in desmin of the cardiac tissues from CM hamsters is significantly higher than that of normal control animals. We also conclude that the phosphorylation of desmin in CM hamster hearts is not a secondary consequence of the CM because PKC phosphorylation stimulated by TPA in cultured normal cardiac cells causes a disorganization of myofibrils, closely mimicking the myofibril disarray patterns observed in CM hamster hearts. Because desmin is a major intermediate filament protein in cardiac tissues, our results indicate that disassembly of desmin filaments, which could be caused by PKC-mediated phosphorylation, may be a factor in myofibril disarrays in CM cells and that the intermediate filament protein, desmin, plays an important role in maintaining myofibril alignment in cardiac cells. However, the possibility cannot be excluded that other sarcomeric proteins, in particular, troponin T, tropomodulin, and myosin-binding C protein, may also contribute to myofibril disarray in the CM hearts. Furthermore, it will be interesting to explore the roles of different PKC isoforms, especially PKC-α and PKC-ε, on desmin phosphorylation and myofibril disarray in CM hearts.

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