

# Contractile Activity and Cell-Cell Contact Regulate Myofibrillar Organization in Cultured Cardiac Myocytes

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**Abstract.** Adult feline ventricular myocytes cultured on a laminin-coated substratum reestablish intercellular junctions, yet disassemble their myofibrils. Immunofluorescence microscopy reveals that these non-beating heart cells lack vinculin-positive focal adhesions; moreover, intercellular junctions are also devoid of vinculin. When these quiescent myocytes are stimulated to contract with the  $\beta$ -adrenergic agonist, isoproterenol, extensive vinculin-positive focal adhesions and intercellular junctions emerge. If solitary myocytes are stimulated to beat, an elaborate series of vinculin-positive focal adhesions develop which appear to parallel the reassembly of myofibrils. In cultures where neighboring myocytes reestablish cell-cell contact, myofibrils appear to reassemble from the fascia adherens rather than focal contacts. Activation of beat-

ing is accompanied by a significant reduction in the rate of total and cytoskeletal protein synthesis; in fact, myofibrillar reassembly, redevelopment of focal adhesions and fascia adherens junctions require no protein synthesis for at least 24 h, implying the existence of an assembly competent pool of cytoskeletal proteins. Maturation of the fascia adherens and the appearance of vinculin within Z-line/costameres, does require de novo synthesis of new cytoskeletal proteins. Changes in cytoskeletal protein turnover appear dependent on  $\beta$  agonist-induced cAMP production, but myofibrillar reassembly is a cAMP-independent event. Such observations suggest that mechanical forces, in the guise of contractile activity, regulate vinculin distribution and myofibrillar order in cultured adult feline heart cells.

THE adult mammalian myocardium generates and is subject to a myriad of mechanical forces from beat to beat. Fluctuations in mechanical load are implicated in regulating the expression and turnover of myofibrillar proteins and the maintenance of the structural integrity of the contractile apparatus (Cooper, 1987; Morgan and Baker, 1991). Increases in hemodynamic load that frequently accompany hypertension, for example, enhance the transcription of constitutively expressed myofibrillar genes (e.g., myosin light chain-2) and provoke the re-expression of embryonic cardiac/skeletal muscle-specific protein isoforms (e.g., skeletal  $\alpha$ -actin,  $\beta$ -myosin heavy chain and atrial natriuretic factor) in pressure overloaded hearts (Morgan and Baker, 1991). Conversely, reductions in myocardial work appear to reduce the capacity of myocardial cells to synthesize contractile proteins, suppress the expression of  $\alpha$ -myosin heavy chain and activate the degradation of contractile proteins (Klein et al., 1992). Moreover, mechanically unloaded, yet contracting papillary muscle (Cooper, 1987), and cultured, quiescent neonatal (Samarel and Englemann, 1991; McDonough and Glembotski, 1992) or adult ventricular myocytes (Decker et al., 1990, 1991; Clark et al., 1991) disassemble and/or degrade myofibrillar proteins, promoting a phenotypic dedifferentiation of the heart cell. Mechanically reloading papillary muscle or inducing contractile activity in

cultured myocytes restores myofibrillar organization. Such observations imply that mechanical force is sufficient to modulate myofibrillar gene expression and assembly; however, the subcellular structural elements that mediate myofibrillar reorganization and the intracellular messengers that regulate these processes remain poorly defined at present.

Since the mechanical forces of contraction are propagated across the sarcolemma of adjacent heart cells, the "cytoskeletal network" that links the contractile apparatus to the intercalated disc and costameres represents a likely candidate in modulating the alignment and stability of myofibrils (Terracio et al., 1991; Danowski et al., 1992). Of the cytoskeletal-associated proteins known to be present in these complexes (Burridge et al., 1988; Geiger et al., 1990), vinculin (Otto, 1991) discloses a perinuclear redistribution in cultured, non-beating rabbit heart cells which parallels the disassembly of the contractile apparatus that develops in such preparations (Simpson et al., 1988). This observation raises the question that changes in mechanical activity may regulate the distribution and turnover of vinculin and perhaps other cytoskeletal proteins and ultimately, the structural integrity of the contractile apparatus in the cardiac myocyte. Although cell density, substrate adhesiveness (Ungar et al., 1986; Bendori et al., 1987), and a variety of growth and serum factors

(Ben-Ze'ev et al., 1990; Bellas et al., 1991) are believed to modulate vinculin expression, turnover, and distribution in cultured cells, mechanical force has not been implicated previously in altering the distribution and turnover of this cytoskeletal-associated protein. Consequently, the objective of the present investigation was to determine whether the acquisition of contractile function mediated by  $\beta$ -adrenergic agonists (Clark et al., 1991) would modulate the distribution of vinculin in focal contacts and intercellular junctions and promote the reassembly of the myofibrillar apparatus. Paired experiments were designed to assess whether changes in cAMP production were associated with cytoskeletal protein turnover and myofibrillogenesis that developed in response to isoproterenol-induced beating. The present observations support the contention that activation of the  $\beta$ -adrenergic pathway mediates significant changes in the turnover of cytoskeletal (CS)<sup>1</sup> proteins, but the physical forces generated during contraction are necessary and sufficient to promote cytoskeletal-myofibrillar reorganization.

## Materials and Methods

### Culture of Adult Cardiac Myocytes

Cardiac myocytes were dissociated from adult feline hearts that have been perfused retrogradely with 0.075% collagenase (Class II; Worthington Biochem. Corp., Freehold, NJ) as described previously (Decker et al., 1990; Clark et al., 1991). Heart cells were suspended in serum-free MEM (Northwestern University Media Center, Chicago, IL), seeded onto laminin-coated 35- or 60-mm petri dishes or 18-mm coverslips at a density of  $10^5$  cells/cm<sup>2</sup> and allowed to adhere to the substratum for 1 h. The cells were rinsed with serum-free media and cultured in MEM supplemented with 2 mM Ca<sup>2+</sup>, 25 mM Hepes (Sigma Chemical Co., St. Louis, MO), 5% FBS (Whittaker Bioproducts, Walkersville, MD), 5% NU serum (Collaborative Res., Inc., Bedford, MA) and insulin (100 nM), transferrin (5  $\mu$ g/ml) and selenious acid (5 ng/ml) (Collaborative Research, Inc., Bedford, MA). Cytosine arabinoside (10  $\mu$ M, Sigma Chemical Co.) was added to the culture media to suppress interstitial cell proliferation (Decker et al., 1990).

Feline myocytes could be activated to beat during the first 3 wk of culture by supplementing the culture medium with  $\beta$ -adrenergic agonists or by agents that elevate intracellular cAMP (Clark et al., 1991). Adult heart cells exposed to the  $\beta_1$  agonist, isoproterenol (ISO) commence beating immediately at a rate of 120 beats per minute and maintain this contractile rate for as long as the  $\beta$  agonist is present. In the present investigation, adult myocytes were exposed to culture medium containing 10  $\mu$ M ISO (Sigma Chemical Co.) on day 5 or day 12 of culture. These two culture periods were chosen because no cell-cell contact exists at day 5 while myocytes maintained for 12 d have reestablished intercellular junctions. Addition of the  $\beta$  agonist induced asynchronous beating at day 5 and synchronous beating at day 12. In each instance, the myocytes contracted at identical rates (i.e.,  $122 \pm 18$  beats per minute [ $n = 25$ ]); moreover, the exposure of such beating cultures to the  $\beta_1$  antagonist, propranolol (10  $\mu$ M), high potassium (KCl, 50 mM) or the L-type Ca<sup>2+</sup> channel blocker, nifedipine (10  $\mu$ M), inhibited contraction immediately in both culture preparations (Clark et al., 1991).

### Fluorescence Microscopy

Vinculin and F-actin were visualized in fixed cells by indirect immunofluorescence microscopy (Decker et al., 1990). Myocytes were rinsed twice in serum-free MEM, fixed for 10 min in 4% paraformaldehyde buffered with PBS at pH 7.4, washed in PBS and extracted for 10 min in absolute acetone at  $-20^\circ\text{C}$ . Antibodies were diluted in PBS plus 0.1% BSA and coverslips were incubated at  $37^\circ\text{C}$  for 30 min in a moist chamber. Three different anti-vinculin antibody preparations were employed to visualize this cytoskeletal-associated protein and included: (a) mouse monoclonal anti-

vinculin (1:100; ICN Biomedicals, Costa Mesa, CA) (Geiger et al., 1985); (b) rabbit polyclonal anti-vinculin (1:100; Terracio et al., 1991); and (c) rabbit polyclonal anti-vinculin (1:50; Transformation Research, Framingham, MA). No differences in vinculin distribution were detected by use of these different anti-vinculin antibody preparations. Coverslips were sequentially incubated with normal goat serum (1:50; Nordic Immunologicals Laboratories, Capistrano Beach, CA), anti-vinculin antibody and rhodamine-phalloidin (1:50; Molecular Probes, Inc., Eugene, OR) and counterstained with fluoresceinated goat anti-mouse or goat anti-rabbit antibodies (1:100; polyclonal goat anti-mouse; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Coverslips were washed in several changes of PBS and mounted in Aquamount (Lerner Laboratories, New Haven, CN) plus 10 mg/ml DABCO (1,4-diazabicyclo [2.2.2]-octane, 97%; Aldrich Chemical Co., Milwaukee, WI) to slow fluorescence quenching. The stained cells were observed with a Leitz Orthoplan microscope equipped with a 75 W Xenon light source and a Vairo-Orthomat camera system.

Vinculin-positive focal contact area was quantitated in the adhesion plaques of quiescent and beating 7-d-old myocytes by integrating the area of the interface between the basal cell surface and the substratum displaying vinculin-positive fluorescence. 35-mm negatives were projected onto a digitizing pad and the boundaries of the adhesion plaque were defined as that area subtended by the sarcolemma and the most distal, intact rhodamine-phalloidin positive I-band (see Fig. 2). After the adhesion plaque was traced, vinculin-positive focal contact area was measured and expressed as nm<sup>2</sup>/ $\mu$ m<sup>2</sup> adhesion plaque  $\pm$  SEM. At least 50 negatives were digitized for each experimental protocol. Significant differences were assessed using a one-way blocked analysis of variance with post-hoc analysis employing the Student-Newman-Kuels multiple range test.

### Electron Microscopy

The organization of the contractile apparatus and the structure of reassembled junctional complexes was assessed by transmission EM. Cultured myocytes were preserved in situ for 2–4 h with 2% glutaraldehyde (EMS, Inc., Fort Washington, PA) buffered in 0.1 M sodium cacodylate (pH 7.4) at room temperature. Dishes were rinsed in cacodylate buffer plus 7.5% sucrose, postfixed in 2% osmium tetroxide (EMS, Inc.) for 1 h and stained in aqueous 2.5% uranyl acetate, pH 6.0, to enhance membrane contrast (Decker et al., 1990). Cells were embedded in Medcast and en face sections were cut, stained, and thin sections were viewed and photographed with a JEOL 100CX electron microscope (JEOL USA, Inc., Peabody, MA).

### Protein Metabolism

Single and double isotopic labeling protocols were used to estimate the accumulation of leucine in cytoskeletal (CS) protein and to monitor the relative turnover rate of selected proteins in cultured myocytes exposed to catecholamine, respectively. The amount of leucine incorporated into actin, myosin heavy chain (MHC) and vinculin was derived from cultures labeled continuously with 0.2  $\mu$ Ci/ml of L-[4,5-<sup>3</sup>H] leucine (specific activity  $\sim$ 120 Ci/mM; Amersham Corp., Arlington Heights, IL) from day 1 of culture. Cultures were rinsed five times in ice-cold, serum-free MEM, extracted in low salt buffer containing 40 mM NaCl, 20 mM Na<sub>2</sub>PO<sub>4</sub>, 0.1 mM MgCl<sub>2</sub>, 0.1 mM DTT, 0.1 mM EGTA, and 1% Triton X-100 and sonicated for 30 s. An aliquot was removed to measure total protein (Lowry et al., 1951), and the balance of the low salt extract was dried, solubilized in 62.5 mM Tris HCl, pH 6.8, containing 5% (vol/vol) 2-mercaptoethanol, 8% (wt/vol) SDS and 10% (wt/vol) glycerol, heated to  $100^\circ\text{C}$  for 15 min. A portion was counted for total [<sup>3</sup>H]leucine incorporation (dpm/culture) while CS proteins were partially purified from another aliquot on a 4–12% vertical gradient SDS-PAGE slab gel prepared using the Laemmli buffer system (Laemmli, 1970). Gels were stained with Coomassie brilliant blue  $\sim$ 250R, destained and then bands previously identified as MHC, vinculin,  $\alpha$ -actinin, desmin, and actin by co-electrophoresis of purified standards or by Western blotting (see Fig. 11) were minced thoroughly and hydrolyzed in 6 N HCl for 24 h at  $110^\circ\text{C}$ . Amino acids were separated by polyamide thin layer chromatography, and the specific radioactivity of [<sup>3</sup>H]leucine in total and cytoskeletal protein was determined by radioassay after derivatization of amino acids with [N-methyl-<sup>14</sup>C] dimethylamino-naphthalene-5-sulphonyl chloride (specific activity  $\sim$ 50 mCi/mM; Amersham Corp.) before separation (Clark et al., 1993). The specific radioactivity of total leucine in danylated protein(s) was expressed in dpm per nanomole. Dividing the leucine incorporation in total or selected protein(s) (dpm/culture) by the specific radioactivity of leucine (dpm/nmol) in each protein(s) yielded the total amount of labeled leucine in those proteins. Such values represent a sensitive method of determining the relative quantities of CS proteins synthesized

1. Abbreviations used in this paper: CHX, cycloheximide; CS, cytoskeletal; ISO, isoproterenol; MHC, myosin heavy chain; P/C, pulse/continuous; PKA, protein kinase A; PKC, protein kinase C.

in the cultured heart cells; total and CS protein content was expressed in nanomoles of leucine.

Relative changes in the turnover of CS protein were evaluated using a pulse/continuous (P/C) kinetic protocol. Cultures were biosynthetically labeled continuously from day 1 with 0.1  $\mu\text{Ci/ml}$  of  $[\text{U-}^{14}\text{C}]$ leucine (specific activity  $\sim 300 \text{ mCi/mM}$ ; Amersham Corp.) and during the last 4 h of the experiment, each plate was pulse labeled with 2.5  $\mu\text{Ci/ml}$  of  $[\text{4,5}^3\text{H}]$  leucine. Cultures were either rinsed and harvested for SDS-PAGE analysis as described above or chased for up to 96 h in complete culture medium supplemented with 2 mM cold leucine to prevent the reincorporation of radiolabeled leucine released as a result of proteolysis. Total protein and individual gel band extracts were counted for their  $[\text{3H}]$  and  $[\text{14C}]$  leucine content and expressed as a ratio of  $[\text{3H}] \text{ dpm}/[\text{14C}] \text{ dpm}$ . The P/C ratio was used to measure relative changes in the synthesis and degradation of cardiac proteins in response to each experimental intervention. P/C ratios were expressed as means  $\pm$  SEM and significant differences in relative rates of synthesis and/or degradation were statistically analyzed by a one-way or two-way analysis of variance followed by Student-Neuman-Keuls multiple range test.

### cAMP Assay

Adenosine 3',5'-cyclic monophosphate (cAMP) content of myocyte cultures was determined using a radioimmunoassay kit supplied by Amersham Corp. Monolayers were rapidly rinsed in ice-cold serum-free MEM, extracted with 1 ml of ice-cold 6% TCA and homogenized. After centrifugation and ether extraction, the supernatants were lyophilized and resuspended in 1 ml of 50 mM acetate buffer, pH 6.0. Extracts were frozen at  $-80^\circ\text{C}$  and assayed at a later date for protein according to the Lowry protocol (Lowry et al., 1951). Equal aliquots (100  $\mu\text{l}$ ) of the extract were combined with the primary anti-cAMP antibody and  $[\text{125I}]$  cAMP, vortexed and incubated for 3 h at  $4^\circ\text{C}$ . An excess (500  $\mu\text{l}$ ) of the second antibody (Amerlex-M) was added to the incubation media, vortexed and incubated for 10 min at room temperature ( $21^\circ\text{C}$ ). The samples were centrifuged at 1,500 g for 10 min, the supernatant was decanted, drained, and then the tubes were counted in a LKB 1275 (LKB Inc., Paramus, NJ) minigamma counter. cAMP content was expressed in picomoles per mg protein  $\pm$ SEM. All cultures were incubated in the presence of 10  $\mu\text{M}$  1-methyl-3-isobutylxanthine (Sigma Chemical Co.) to inhibit cAMP phosphodiesterase activity and prevent cAMP degradation. The specificity of ISO-induced cAMP production was monitored by incubating paired cultures in the presence of the  $\beta_1$ -antagonist, propranolol. Changes in cAMP content were statistically evaluated in a fashion identical to the protein turnover experiments.

## Results

### Redistribution of Actin and Vinculin in Quiescent Heart Cells

One day after isolation and attachment to a laminin-coated substratum, primary cultures of adult feline cardiac myocytes consist predominantly of solitary, binucleated cells that display a rod-shaped configuration (Fig. 1 A). Furthermore, such cell preparations remain quiescent unlike their neonatal counterparts which display spontaneous contractility. The actin-based myofibrillar apparatus retains its structural registry (Fig. 1 B) and anti-vinculin antibodies stain the cells along the Z-lines in a regular repeating array that typifies the distribution of this cytoskeletal-associated protein in the intact heart (Fig. 1 C). In addition, a prominent, amorphous anti-vinculin staining pattern (Fig. 1 C) is apparent at the distal ends of the cells and at irregular intervals along their lateral borders where the myocytes display a "stair-step" morphology which is consistent with the reported distribution of the fascia adherens junction in the intact, feline myocardium (Fawcett and McNutt, 1969).

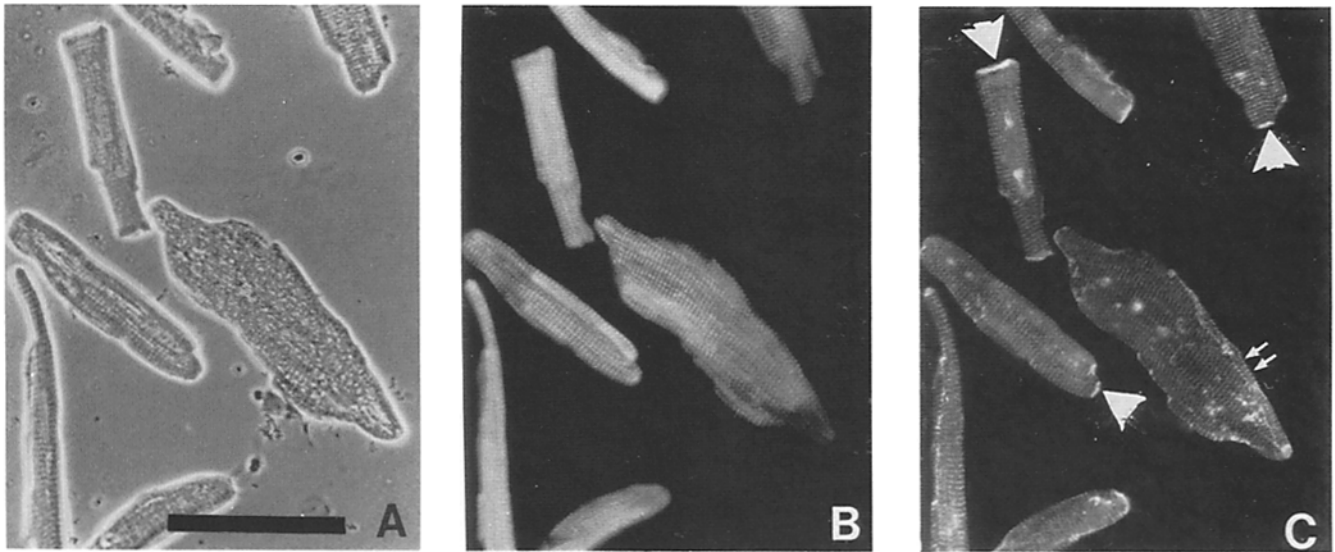
After 7 d in culture, feline myocytes display a cylindrical, rod-like region from which rounded, lamellipodia-like membrane processes project from either end of the cell. A deterioration in the lateral registry of the myofibrillar ap-

paratus develops in parallel with a redistribution of vinculin from these regions (Fig. 2). Vinculin becomes depleted from the Z-lines and assumes a diffuse pattern just distal to the contractile apparatus (Fig. 2, A and B). Interference reflection microscopy illustrates the presence of a small population of punctate reflection images and a larger more amorphous gray reflection in the central portion of the lamellipodium but evidence of classic focal adhesions is lacking in live heart cells (Fig. 2 C). An actin cable becomes oriented subjacent to the sarcolemma with smaller cables emerging from and apparently terminating at the edge of the plasma membrane (Fig. 2, A). Although no vinculin is associated with the prominent actin cable, vinculin does colocalize with the smaller subsarcolemmal cables. Interference reflection microscopy further suggests that this site is in close contact with the substratum (Fig. 2, A-C). Electron micrographs reveal that the vinculin-negative actin cable bears some resemblance to myofibrils for they exhibit electron-dense elements reminiscent of Z-lines; other pleomorphic Z-like densities also appear scattered randomly throughout these lamellipodia (Fig. 2 D).

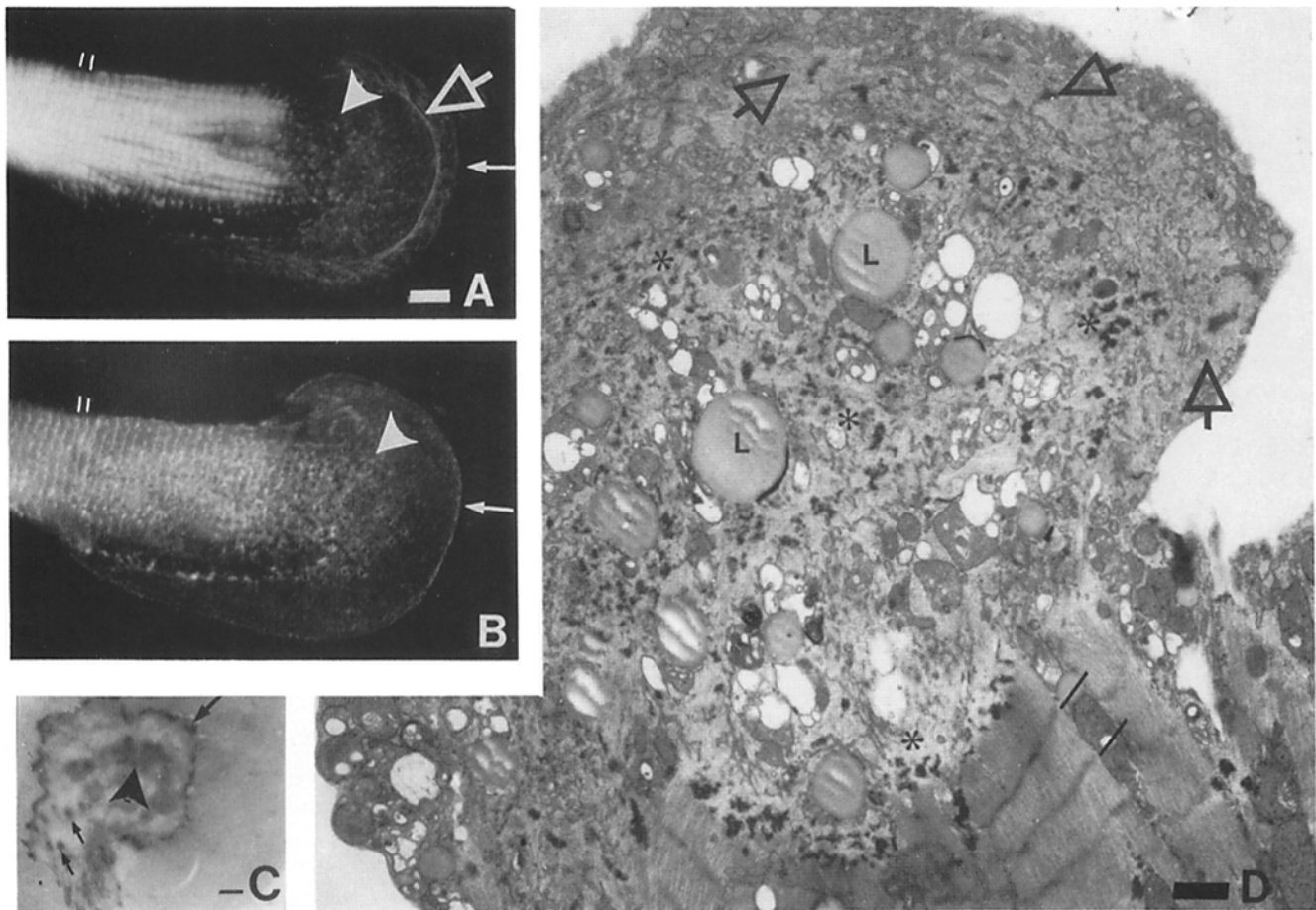
While adult myocytes don't reestablish cell-cell contact during the first week of culture, by the second week continued cell spreading transforms the rod-shaped myocytes into cells displaying a flattened, nonpolarized configuration (Fig. 3). Myofibrillar order is markedly disrupted (Fig. 3 A) in these nonbeating heart cells with "dedifferentiated" myofibrils possessing few thick and thin filaments; Z-line remnants appear to represent the predominant contractile element present in these myocytes (Fig. 3, C and D). Neighboring heart cells develop extensive zones of intercellular contact and assemble desmosomes and gap junctions (Fig. 3, C and D); however, anti-vinculin antibodies fail to divulge any vinculin-positive structures within these zones of intercellular contact (Fig. 3 B) and there is no structural evidence of nascent fasciae adherentes or actin thin filaments interacting with this newly established junctional complex (Fig. 3 C). En face EM images illustrate that little myofibrillar actin is anchored to any definable sarcolemmal structure; moreover, even though desmosomes reassemble, intermediate filaments appear haphazardly distributed in the subjacent cytoplasm (Fig. 3 C). Anti-vinculin staining is confined to perinuclear regions of the myocyte, and double-label fluorescence microscopy further documents that little of this diffuse, anti-vinculin immunofluorescence is associated with the Z-lines of the remaining myofibrils (Fig. 3, A and B) nor are vinculin-positive costameres readily apparent in these quiescent heart cells (Fig. 3 B).

### Contraction-mediated Myofibrillar Reassembly

$\beta$ -adrenergic agonists activate cultured myocytes to beat within seconds, regardless of culture age (Clark et al., 1991). The acquisition of contractile function dramatically modulates the distribution of actin and vinculin in these myocytes. Solitary myocytes that are cultured for 5 d and activated to beat for 24-48 h display a rod-like region with lamellipodia that project from either end like their nonbeating counterparts. However, these spread membrane processes acquire a distinctive angular shape, displaying well-ordered myofibrils and extensive deposits of vinculin on their basal surfaces (Fig. 4, A and B). These deposits of vinculin are variable in size and shape, ranging from a slender and extremely elon-

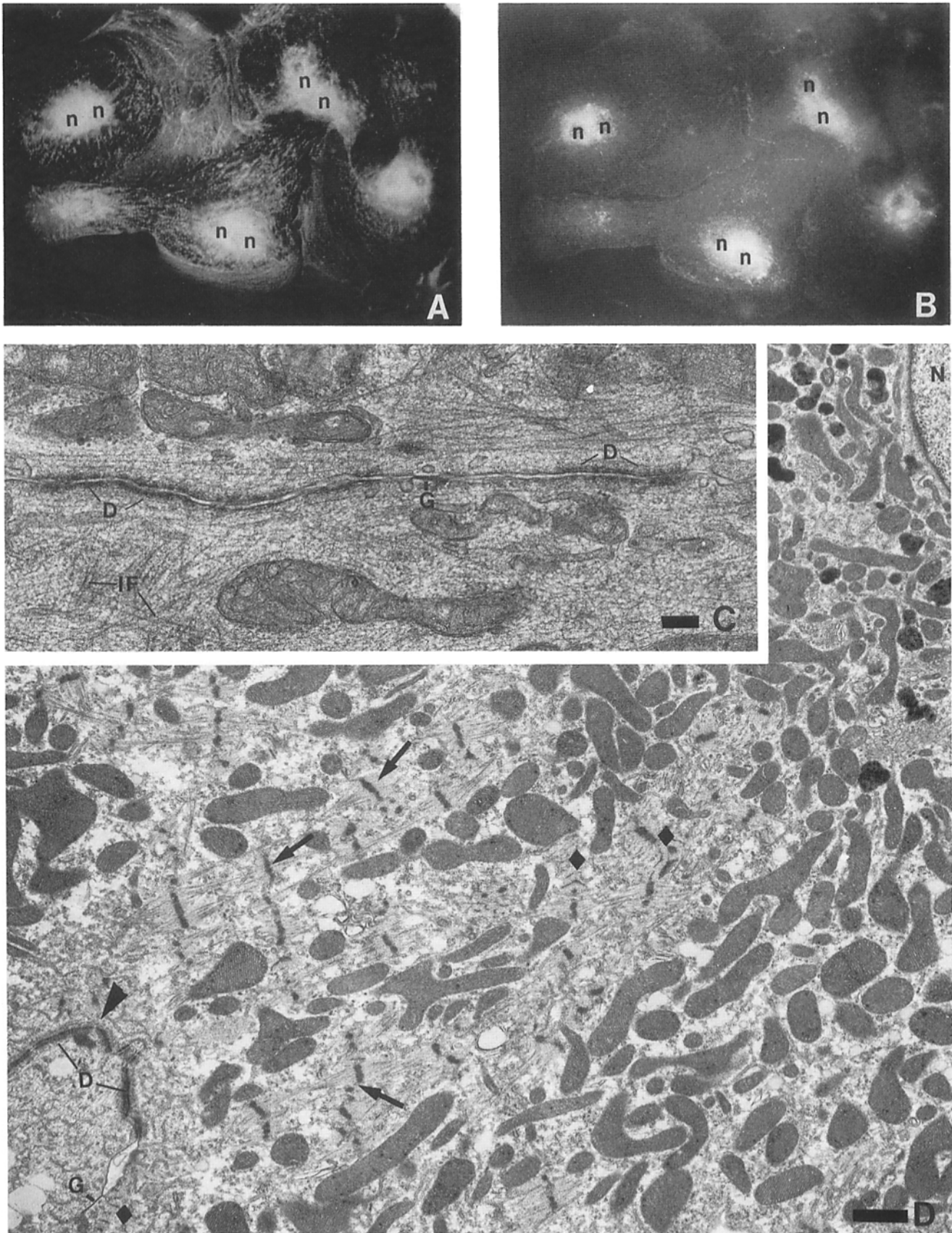


**Figure 1.** Adult feline myocytes cultured for 1 d. Phase microscopy (A). Rhodamine-phalloidin labels actin-positive I bands of myofibrils (B) while vinculin antibodies (C) are restricted to Z-lines (arrows) and the remnants of the intercalated discs (arrowheads). Bars, 50  $\mu\text{m}$ .

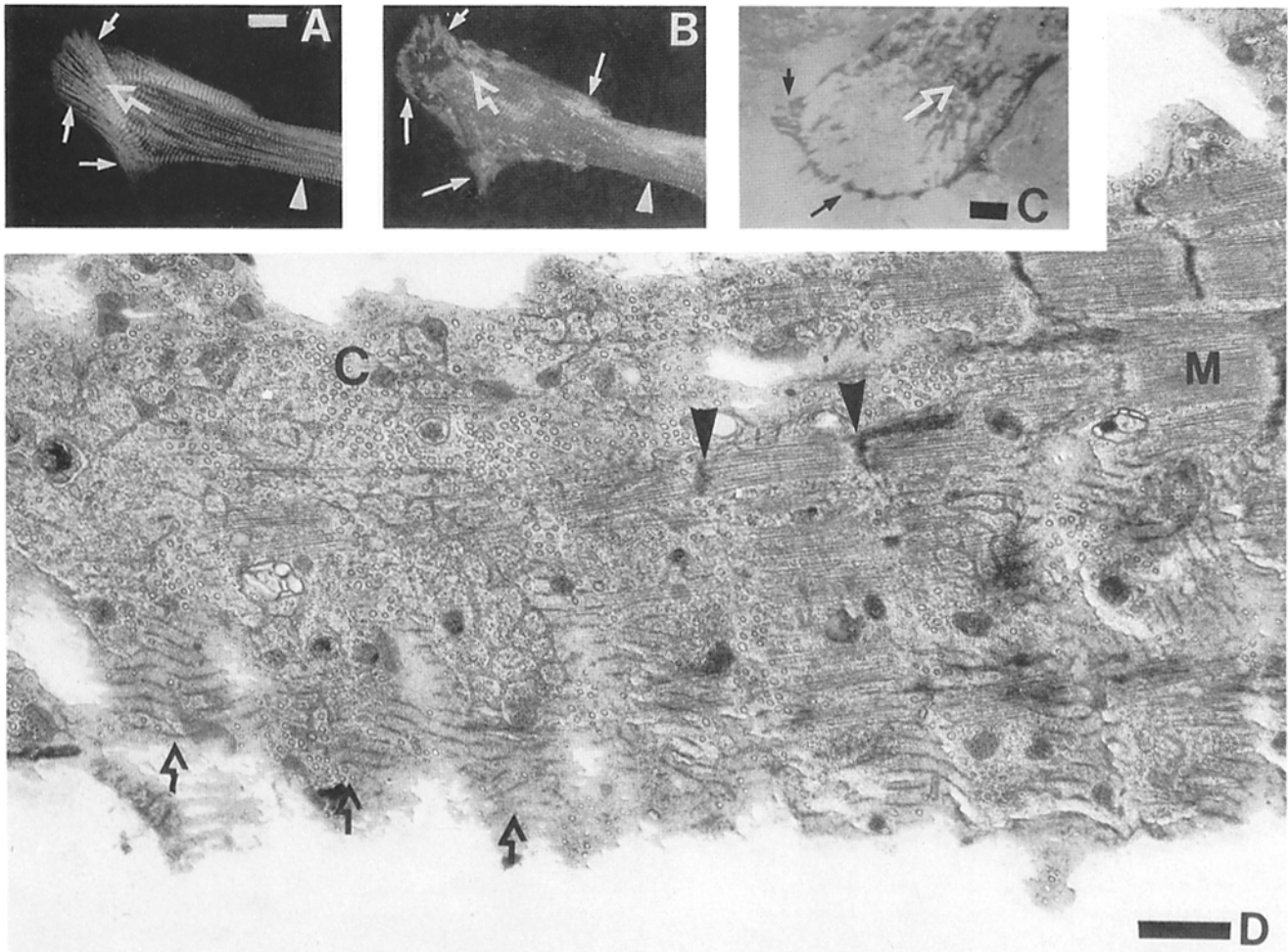


**Figure 2.** Distal adhesion plaque of a nonbeating heart cell cultured for 7 d. Actin (A) and vinculin (B) colocalize only at the edge of the sarcolemma (arrows); IRM (C) images reveal a continuous close contact at this site. Actin and vinculin display unique staining patterns (arrowheads) in the central region of the plaque. IRM, however, reveals only a dull gray interference pattern (arrowhead) in this zone while punctate focal contacts (small arrows) are rare (C). Thin sections reveal that actin cables (A, open arrowhead) exhibit a few Z-like densities (D, open arrowheads). Haphazardly distributed Z-like densities (asterisks) are observed at the transition zone between intact myofibrils (lines) and the plaque. L, lipid. Bars: (A, B, and C) 10  $\mu\text{m}$ ; (D) 1  $\mu\text{m}$ .



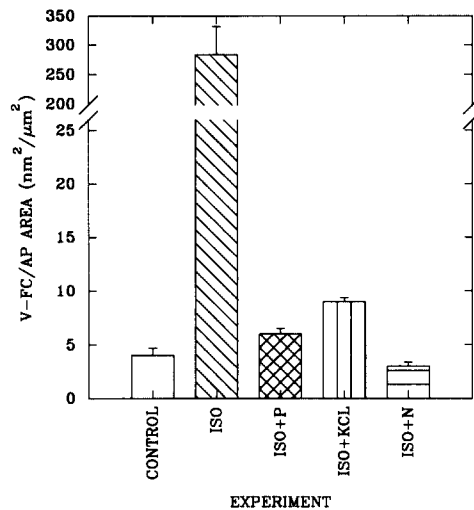


**Figure 3.** Myofibrils in quiescent myocytes cultured for 2 wk. Rhodamine-phalloidin-positive myofibrils (A) appear disorganized and actin (A) and vinculin (B) assume a perinuclear (n) location. En face thin sections of the perinuclear (N) areas reveal marked myofibril disruption (arrows). The intercalated disc (arrowhead) is reestablished but only desmosomes (D) and gap junctions (G) are apparent (C and D). No well-organized actin arrays terminate at these junctions (C and D) and intermediate filaments (IF) do not appear associated with desmosomes (C). Bars: (A and B) 10  $\mu\text{m}$ ; (C) 0.1  $\mu\text{m}$ ; (D) 1  $\mu\text{m}$ .



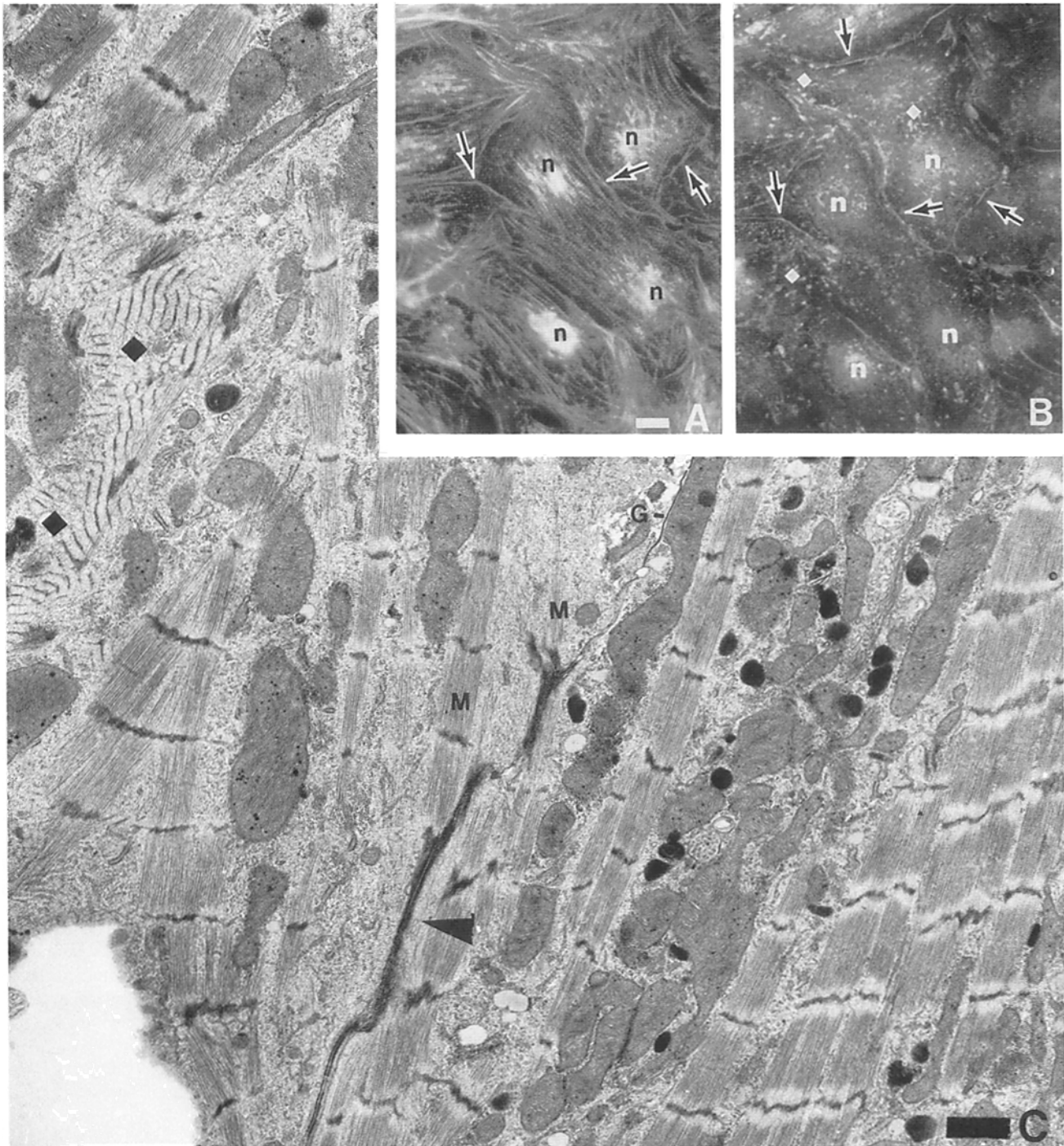
**Figure 4.** A portion of an adhesion plaque of a 7-d-old myocyte stimulated to contact on day 6 of culture. Beating induces myofibril reassembly (A) and F-actin becomes anchored to the sarcolemma (arrows) and at vinculin-positive focal adhesions (arrowhead) (A and B). IRM (C) reveals the dark reflection patterns of sarcolemmal (arrows) and central focal adhesions (arrowhead) in a living cell correspond to vinculin-positive zones in B. An en face profile of a distal adhesion plaque illustrates numerous leptomeris (open arrowheads) that appear associated with terminating myofibrils (M). Z-lines (arrowheads); M, myofibril; C, caveolae. Bars (A and B) 20  $\mu\text{m}$ ; (C) 10  $\mu\text{m}$ ; (D) 1  $\mu\text{m}$ .

gated configuration to a more compact pyramidal shape that resemble the focal adhesion of a cultured fibroblast (Fig. 4 B). The vinculin-positive deposits appear to coincide with the focal contacts visualized in living heart cells with interference reflection microscopy (Fig. 4 C). The basal surface area displaying these vinculin-positive focal contacts increases  $\sim 70$ -fold over that documented in nonbeating heart cells (Fig. 5). Moreover, the  $\beta$  antagonist, propranolol, membrane depolarization, or L-type  $\text{Ca}^{2+}$  channel blockade suppresses ISO-induced contractile activity and precludes the redistribution of vinculin (Fig. 5). The finely stained network of non-myofibrillar actin cables that characterize the lamellipodia of quiescent cells are no longer discernable in the processes of beating cells (Figs. 2 A vs. 4 A). Instead, reassembled myofibrils appear to be anchored directly to



**Figure 5.** Vinculin positive focal contact (V-FC) area derived from digitized fluorescent images is expressed as  $\text{nm}^2$  occupied by vinculin per  $\mu\text{m}^2$  of adhesion plaque (AP) basal surface area (i.e.,  $\text{nm}^2/\mu\text{m}^2$ ). Each value represents the mean ratio  $\pm$  SEM derived from 50 cells. ISO-induced beating increases vinculin-positive fo-

cal adhesions significantly ( $P < 0.001$ ) when compared to paired quiescent myocytes (control) or those heart cells cultured in the presence of ISO-supplemented with propranolol (P), KCl, or nifedipine (N).



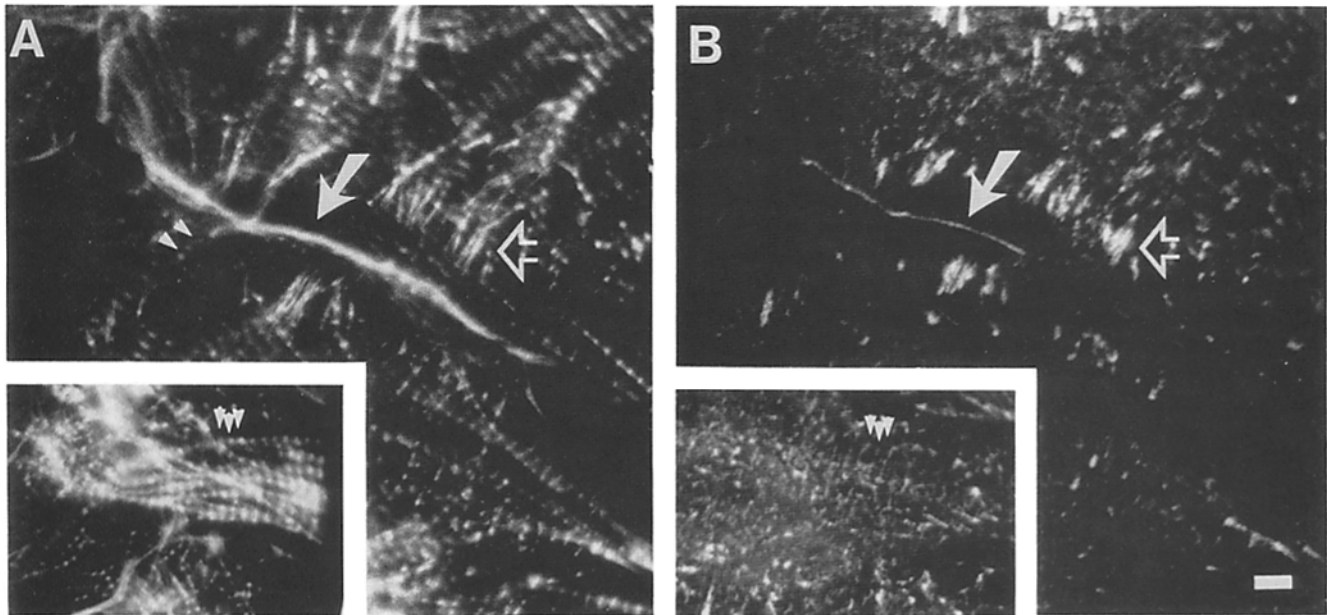
**Figure 6.** 2-wk-old myocytes stimulated to beat on day 13 of culture. Myofibrils reacquire their linear orientation and actin cables (arrows) appear at sites of cell–cell contact (A). Vinculin (B, arrows) colocalizes with the actin cables (A) and numerous small focal adhesions (◆) develop. Diffuse perinuclear (n) vinculin (B) deposits and F-actin (A) are also present. EM images (C) reveal junctional complexes (arrowhead) with a few myofibrils (M) terminating at adherens-like dense plaques. Leptomers (◆); G, gap junction. Bars (A and B) 20  $\mu\text{m}$ ; (C) 1  $\mu\text{m}$ .

large focal adhesion complexes via uniformly stained actin cables (Fig. 4, B and C). Electron micrographs derived from en face thin sections depict the site where myofibrils appear to lose their Z-lines in these zones of focal contact (Fig. 4 D).

Reestablishment of cell–cell contact alters the pattern of myofibrillar reassembly observed in solitary, beating heart cells. Myocytes that have been beating for 24 h develop three

discrete types of vinculin-positive structures in response to catecholamine exposure. The first-class of structures is represented by prominent bands of vinculin that stain the plasma membrane in regions of cell–cell contact and are observed to be coincident with non-myofibrillar actin cables (Fig. 6, A and B); however, only a small number of myofibrils appear to terminate in these vinculin-positive junctions. Fine struc-





**Figure 7.** A junctional complex between two 14-d-old myocytes that have been beating for 24 h. A continuous actin cable (*A*, white arrow) colocalizes with vinculin (*B*, white arrow) in the zone of cell–cell contact. Few myofibrils (small arrowheads) appear to terminate at the junction (*A*), rather most are anchored to vinculin-positive (*B*) focal contacts (open arrowheads). Z-line/costamere (small arrowheads) vinculin staining (*B*, inset) is only apparent in regions where myofibrillar registry is reestablished (*A*, inset). Bar, 10  $\mu\text{m}$ .

tural images of these zones of cell–cell contact confirm the existence of long stretches of junctional complex which parallel the reassembled myofibrils (Fig. 6 *C*). The junction is characterized by a dense submembranous plaque but few myofibrils appear associated with this dense plaque. The junction is not present in similar regions of adjacent, nonbeating heart cells (Figs. 3, *C* and *D* vs. 6 *C*) nor does it develop when beating is prevented by including KCl or propranolol in ISO-supplemented culture medium. At this juncture the myofibrillar apparatus reacquires a linear polarity when compared to its age-matched, nonbeating counterpart (Figs. 3 vs. 6). In addition, a significant quantity of vinculin and F-actin can be visualized in the perinuclear regions of these contracting myocytes (Fig. 6, *A* and *B*). The second class of vinculin-containing structures consists of a population of diffusely scattered, pyramidal-shaped focal contacts (Figs. 6 *B* and 7 *B*). The size and complexity of these adhesion sites do not compare with the focal contacts that develop in the solitary 7-d-old beating myocytes (Figs. 4 *B* vs. 6 *B*), but the majority of reassembled myofibrils appear anchored at these sites (Fig. 7, *A* and *B*). The final class of vinculin-containing structures appears to reflect the reassociation of this protein with myofibrillar Z-lines and/or costameres. This anti-vinculin staining pattern is rarely encountered in cells that have been beating for 24 h. When it is observed, it is characterized by a periodic, punctate distribution (Fig. 7 *B*, inset) that resembles the distribution of vinculin observed at the Z-lines and costameres of the intact developing heart (Tokuyasu, 1989). The most extensive regions of costameric or Z-disc vinculin staining appear to occur where the reassembling myofibrils are in close apposition with the lateral and apical sarcolemma and where I-bands display a relatively high degree of lateral registry (Fig. 7, *A* and *B*, insets).

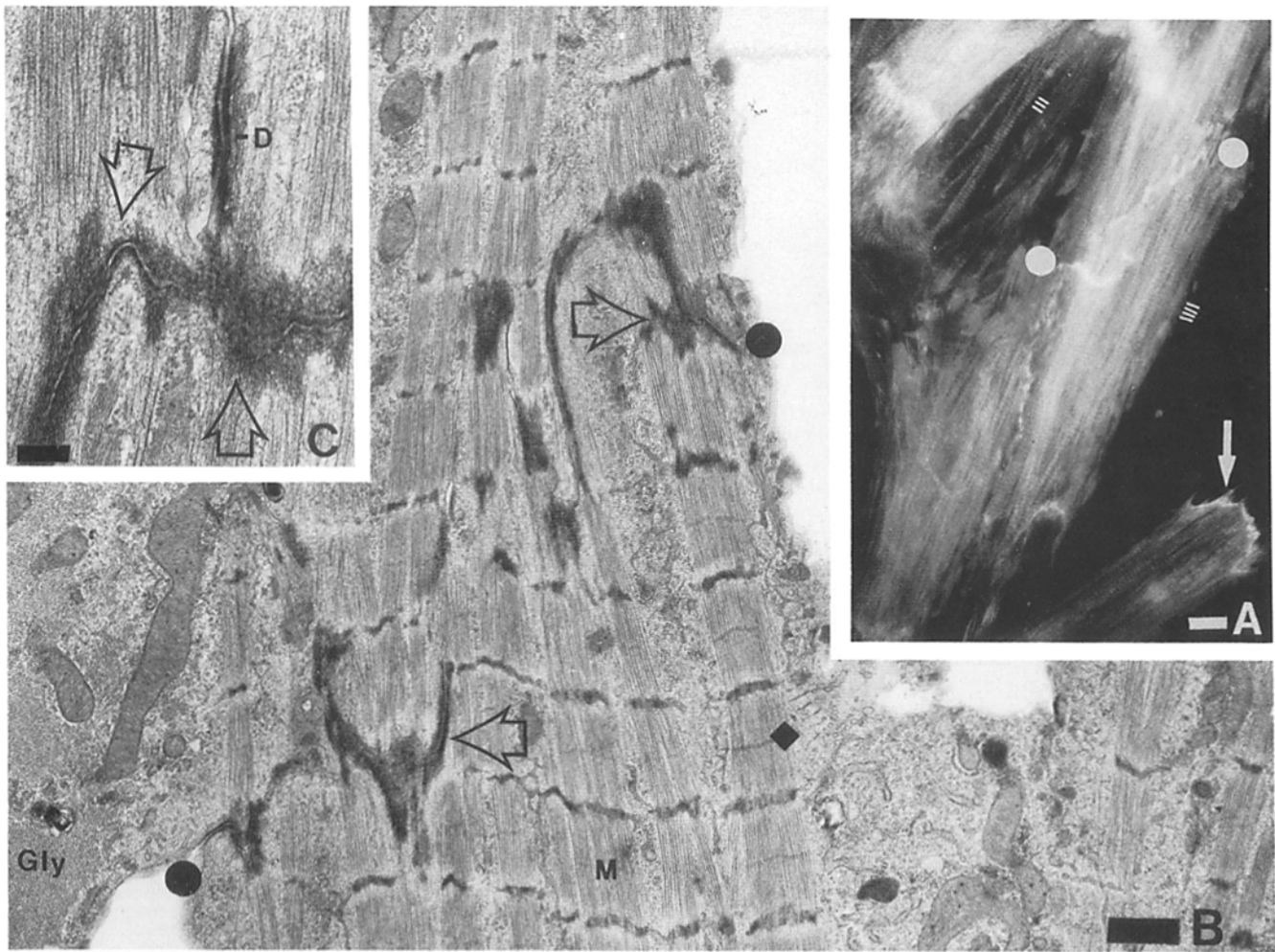
Between 24 and 48 h after exposure to ISO, significant

“remodeling” of the “putative” intercalated disc and the contractile apparatus is apparent. In most instances the intercalated disc assumes a location at the distal ends of adjacent myocytes (Fig. 8) and can be easily recognized by its strong positive vinculin signal (Fig. 8 *A*). Where 24 h earlier most myofibrils appear to be anchored into small focal adhesions, at 48 h the majority of the myofibrils now appear to terminate directly into the fascia adherens. Moreover, vinculin-positive focal adhesions are noted only at sites where a myocyte process does not establish contact with a neighboring heart cell (Fig. 8 *A*). Here actin colocalizes with focal adhesions that reveal vinculin. While few myofibrils display vinculin-positive Z-lines at 24 h, most Z-lines disclose this protein after 48 h of ISO treatment, and the perinuclear distribution of vinculin so prominent at 24 h is absent at 48 h (Fig. 8 *A*). At this juncture thin filaments of myofibrils appear to terminate into the subplasmalemmal dense plaques of the reassembled fascia adherens (Fig. 8, *B* and *C*).

#### Turnover of Cytoskeletal Proteins

Since a marked subcellular redistribution of CS proteins accompanies myofibrillar reorganization, CS protein metabolism was monitored in paired myocyte preparations. The objective of these experiments was to acquire new information on the translational mechanisms that regulate the turnover of CS protein. Beating heart cells that were labeled continuously with [ $^3\text{H}$ ]leucine revealed that the amount of leucine incorporated into total protein was reduced slightly after 24 h of ISO treatment, but this decline became insignificant after 48 h of  $\beta$  agonist exposure (Table I). When equal amounts of protein (20  $\mu\text{g}/\text{lane}$ ) were separated on SDS-PAGE gels, no overt changes in the relative amounts of individual CS proteins could be documented in this investigation, regardless of whether the cells were in contact with one





**Figure 8.** Intercalated disc between 14-d-old myocytes beating for 48 h. The disc assumes its classical stair-step morphology (●—●) as contracting heart cells become more linearly oriented (A and B). The myofibrils (M) display a high degree of lateral registry (B) and Z-lines disclose vinculin (≡, A). Dense plaques (open arrows) become well developed and actin thin filaments appear to terminate into these plaques (C). At sites where no cell–cell contact develops, myofibrils attach to vinculin positive focal adhesions (arrow, A). D, desmosome; Gly, glycogen; G, gap junction; (◆) leptomere. Bars: (A) 20 μm; (B) 1 μm; (C) 0.1 μm.

another (i.e., 5–7-d-old vs. 12–14-d-old cultures) or beating (Fig. 9 A). Furthermore, Western blots of vinculin, for example, displayed no significant differences in the relative amounts of blotted protein (Fig. 9 B). The impression derived from these Western blots could be confirmed indirectly when paired gel bands were extracted and assayed for total

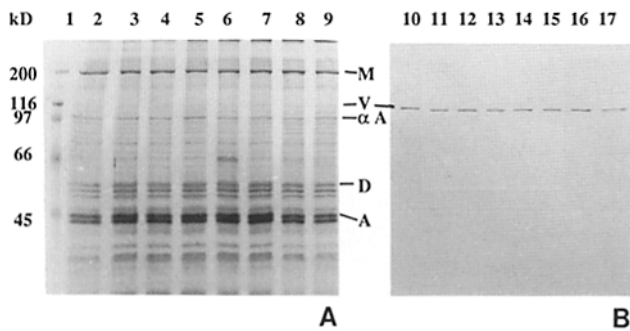
[<sup>3</sup>H]leucine content. Regardless of whether the preparation was beating or quiescent, amounts of protein-bound leucine in actin, MHC and vinculin remained unchanged after 48 h exposure to ISO; moreover, the values were not significantly different from the [<sup>3</sup>H]leucine content of nonbeating control cultures (Table I). However, when β agonist-induced beating

**Table I. Leucine Content of Actin, MHC, and Vinculin in Myocyte Cultures Exposed to ISO**

Time	Protein-bound leucine (nmoles/plate)*							
	ISO (10 μM)				ISO (10 μM) + KCl (50 nm)			
	Total protein	MHC	Actin	Vinculin	Total protein	MHC	Actin	Vinculin
<i>h</i>								
0	169 ± 23	23.2 ± 2.2	29.5 ± 4.1	1.4 ± 0.2	169 ± 23	23.2 ± 2.2	29.5 ± 4.1	1.4 ± 0.2
12	145 ± 12	18.4 ± 2.1	27.6 ± 3.4	1.1 ± 0.4	146 ± 28	19.5 ± 3.7	28.4 ± 3.7	1.3 ± 0.4
24	128 ± 19	22.6 ± 3.3	23.6 ± 4.2	1.8 ± 0.3	157 ± 19	17.8 ± 3.1	22.8 ± 4.1	0.7 ± 0.3‡
48	157 ± 23	24.1 ± 2.8	24.6 ± 2.0	1.7 ± 0.2	153 ± 32	15.9 ± 1.7‡	24.9 ± 4.7	0.9 ± 0.2‡

\* Leucine content was expressed as the mean ± SEM of 6–9 paired preparations derived from cell cultures at each time point.

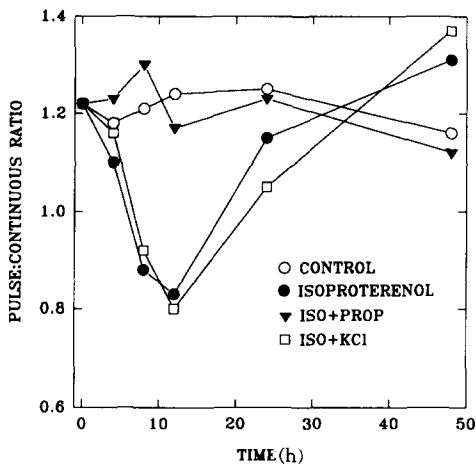
‡ *P* < 0.05 when compared to corresponding time 0 control values.



**Figure 9.** SDS-PAGE gel (A) and Western blot of vinculin (B) from cultured myocytes stimulated to beat with ISO for 0, 12, 24, and 48 h on day 5 (lanes 2–5 and 10–13) or day 12 (lanes 6–9 and 14–17). No apparent changes in the relative amounts of cytoskeletal proteins could be documented in 1- or 2-wk-old cultures, regardless of whether they were beating (lane 3–5 and 7–9) or quiescent (lanes 2, 6 and 10, 15). Similarly, Western blots of vinculin (lanes 10–17) revealed no change in blotted protein nor was any meta-vinculin noted in these cultured myocytes. Lane 1, molecular weight standards. M, myosin heavy chain; V, vinculin;  $\alpha A$ ,  $\alpha$ -actinin; D, desmin; A, actin.

was inhibited with KCl, the leucine content of total cell protein and actin declined slightly while the protein-bound leucine in MHC and vinculin was reduced significantly, especially at 48 h (Table I).

The turnover of total and CS protein was evaluated using a P/C radiolabeling protocol to determine whether unique changes in the synthesis and/or degradation of these proteins were responsible for the fluctuations in CS protein(s) content described above. This double-label technique provides an opportunity to compare and contrast the turnover of long-lived proteins that were labeled primarily with [ $^{14}$ C]leucine



**Figure 10.** Effects of ISO on the synthesis of total cell protein in P/C-labeled heart cells cultured for 14 d. Cultures were exposed to ISO (●), ISO plus propranolol (▼), or ISO and KCl (□) for 48 h with experimental and control (○) cultures assayed at 0, 4, 8, 12, 24, and 48 h. Values represent the mean P/C ratio  $\pm$  SEM, where SEM ranges from 8 to 12% of the mean P/C ratio at each time point; each measurement is derived from 9–12 cultures. ISO and ISO + KCl suppressed total protein synthesis significantly within 8 h ( $P < 0.01$ ) of hormone treatment with P/C values becoming significantly ( $P < 0.05$ ) elevated above control values at 48 h.

**Table II.** Cyclic AMP Content of Cultured Heart Cells Exposed to  $\beta$ -Adrenergic Agonists, Antagonists, and KCl

Time	cAMP Production (pmol cAMP mg $^{-1}$ protein)			
	Control (n = 12)	ISO (n = 12)	ISO propranolol (10 $\mu$ M) (n = 12)	ISO KCl (50 mM) (n = 15)
h				
0	53 $\pm$ 6	—	—	—
0.25	47 $\pm$ 10	157 $\pm$ 23 $^{\ddagger}$	49 $\pm$ 5	141 $\pm$ 12 $^{\ddagger}$
1	55 $\pm$ 5	110 $\pm$ 17 $^{\ddagger}$	61 $\pm$ 5	114 $\pm$ 11 $^{\ddagger}$
24	55 $\pm$ 7	69 $\pm$ 7 $^*$	54 $\pm$ 7	73 $\pm$ 8 $^*$
48	48 $\pm$ 6	63 $\pm$ 8 $^*$	51 $\pm$ 8	67 $\pm$ 6 $^*$

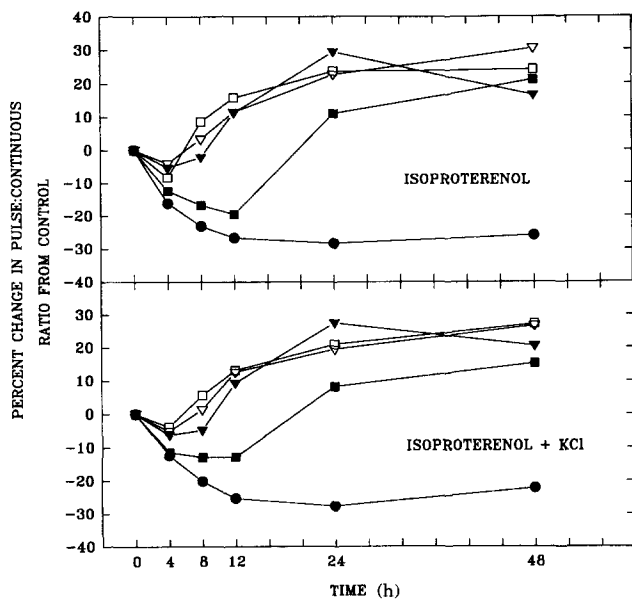
\*  $P < 0.05$  vs. control of equivalent age.

$^{\ddagger}$   $P < 0.01$  vs. control of equivalent age.

n, number of cultures assayed.

with those nascent proteins labeled during the 4 h pulse of [ $^3$ H]leucine after activation of beating with catecholamine. Application of this approach demonstrated that ISO-induced beating at day 5 (data not shown) or day 12 of culture was accompanied by significant depression in total protein synthesis that was evident as early as 4 h after ISO exposure and was sustained for at least 12 h before returning to or above control values at 24 and 48 h, respectively (Fig. 10). Propranolol blocked the decline (i.e., the reduction in [ $^3$ H]leucine incorporation) in the relative rate of protein synthesis through the 48-h period of exposure to the  $\beta$  agonist and antagonist. Like propranolol, KCl (50 mM) also blocked ISO-induced beating; however, changes in the relative rate of protein synthesis displayed similar kinetics to ISO-treated cultures (Fig. 10). KCl, alone, had no influence on protein synthesis over the 2-d interval. Since  $\beta$ -adrenergic receptor occupation induces cAMP production, activates protein kinase A (Gilman, 1987) and provokes beating (Clark et al., 1991), cAMP production was monitored in relationship to myofibrillar reorganization and the turnover of CS proteins. ISO elevated intracellular cAMP levels  $\sim$ threefold within 15 min of exposure to the  $\beta$  agonist (Table II). At 1 h cAMP content was reduced but still remained 40% higher than corresponding control values; only at 24 and 48 h were cAMP levels near control values although, even then, they remained modestly elevated (Table II). Propranolol suppressed the anticipated rise in cAMP in response to ISO while inclusion of 50 mM KCl did not alter synthesis of cAMP in the presence of the  $\beta$  agonist (Table II). Neither KCl or propranolol, alone, altered cAMP levels over the duration of the experiments described above.

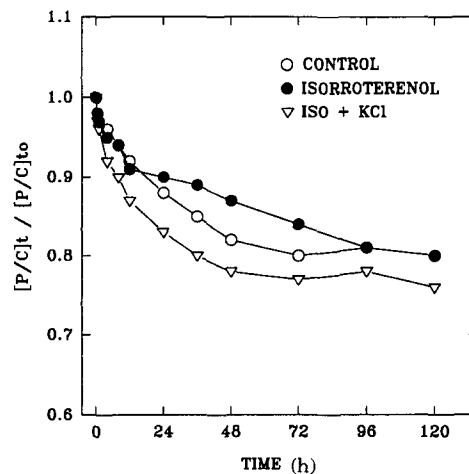
Relative changes in the synthesis of CS proteins was explored to determine whether the kinetics of individual proteins fluctuated in parallel with total myocyte protein or responded uniquely to catecholamine. Within 4 h of exposure to ISO, the P/C ratio of individual CS proteins was reduced to varying degrees. After 12 h, MHC and vinculin P/C ratios remained significantly below corresponding control values, whereas actin,  $\alpha$ -actinin and desmin P/C ratios were elevated 10–15% above control values (Fig. 11). At 24 and 48 h, synthesis of MHC continued to remain depressed  $\sim$ 25% while the P/C ratios for the other CS proteins ranged from 18 to 32% above control values with a marked increase in the incorporation of [ $^3$ H]leucine in vinculin developing between 12 and 24 h after  $\beta$  agonist treatment (Fig. 11). Over



**Figure 11.** Relative synthesis rates of cytoskeletal proteins in 14-day-old feline myocytes cultured in the presence of ISO or ISO plus KCl. Values represent the mean percent change in P/C ratios from the time 0, control P/C ratio. Each value is derived from the SDS-PAGE gel bands obtained from nine separate culture preparations. Cytoskeletal proteins were identified by Western blotting and include myosin heavy chain (●), vinculin (■),  $\alpha$ -actinin (▼), desmin (▽), and actin (□). ISO and ISO + KCl depress the synthesis of V (■) and MHC (●) significantly ( $P < 0.05$ ) within 8 h of treatment. At 12 h, vinculin and MHC synthesis remain depressed ( $P < 0.01$ ), whereas actin,  $\alpha$ -actinin, and desmin synthesis is elevated modestly. All P/C ratios are increased significantly ( $P < 0.05$ ) at 48 h with the exception of MHC which continues to be depressed ( $P < 0.01$ ).

the 48-h period investigated, the inclusion of propranolol completely blocked ISO-induced changes in CS protein synthesis. Conversely, the CS protein P/C ratios mirrored those observed with ISO, alone, when the cultures were partially depolarized with KCl in the presence of the catecholamine (Fig. 11).

Because the amount of any CS protein reflects the difference between its rate of synthesis and degradation, double-labeled, nonbeating and ISO-activated cultures were chased in cold medium to measure CS turnover. The P/C labeling protocol allows for the simultaneous study of the degradation of newly synthesized [ $^3$ H]leucine-labeled protein and long-lived proteins that were labeled with [ $^{14}$ C]leucine. P/C ratios displayed a biphasic pattern of decay for total and CS proteins with [ $^3$ H]leucine-labeled protein being preferentially degraded during the first 48 h of the chase period (Fig. 12). If the chase period was extended for an additional 3 d, the rate of protein degradation slowed significantly, implying the existence of at least two protein pools displaying unique degradation kinetics. ISO slowed the rate of proteolysis in the rapid turnover pool but did not appear to alter the half-life of the slow pool (Fig. 12). Conversely, cultures exposed to ISO and KCl accelerated the breakdown of the rapidly labeled protein pool (Fig. 12). The kinetics of MHC degradation resembled that displayed by total protein but vinculin breakdown was accelerated in the ISO-treated heart cell cultures, especially during the first day of exposure to catechol-



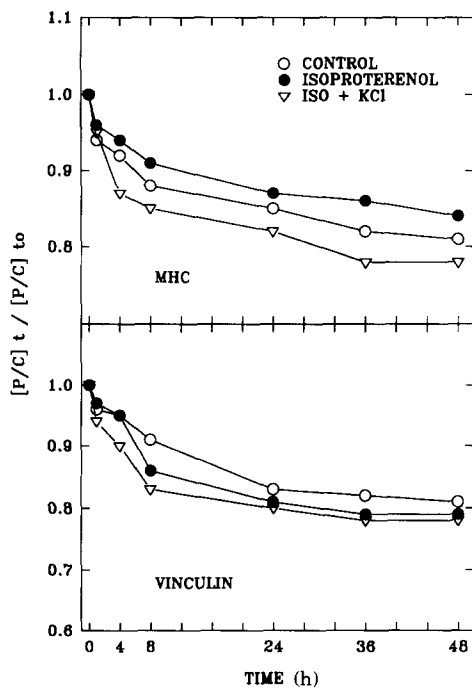
**Figure 12.** Relative turnover of total protein in double labeled (P/C) cultures exposed to ISO (●) or ISO + KCl (▽) and chased for 5 d in cold medium. Each value is expressed as the [P/C] at any time  $t$  divided by the [P/C] at time zero and is derived from 4–6 cultures. ISO appears to slow the breakdown of primarily  $^3$ H-protein while ISO + KCl enhances proteolysis when compared to control (○) cultures.

amine. In contrast, cultures supplemented with  $\beta$  agonist and KCl displayed increased rates of MHC and vinculin proteolysis over the 24 h of the chase period (Fig. 13).

Since newly synthesized CS protein was preferentially degraded in quiescent and contracting heart cells, the question arose whether this nascent protein was essential to the early phases of myofibrillar reorganization. To answer this query, myocyte cultures were activated to beat with catecholamine in the presence of sufficient cycloheximide (100  $\mu$ g/ml) to inhibit protein synthesis  $>90\%$ . In addition to suppressing total protein synthesis, the synthesis of MHC and vinculin was inhibited  $\sim 95\%$  when cycloheximide (CHX) was present in the culture medium from 24 to 48 h after ISO activation (Table III). Pulse/chase experiments further demonstrated that the rate of proteolysis was not altered in cultures treated with CHX over the same interval (Table III). CHX was administered only over the first 24 h of culture or medium was supplemented with the antibiotic from 24–48 h after treatment with catecholamine, because some toxicity developed after 36 h of continuous exposure to the drug. Nevertheless, CHX failed to affect the contractile properties of the myocyte cultures, with heart cells beating at a rate of  $124 \pm 18$  beats per min ( $n = 10$ ) after a 24 h exposure to CHX and ISO; moreover, if CHX treatment was delayed 24 h, contractile activity also remained unchanged ( $116 \pm 18$  beats per min). In those catecholamine-treated cultures exposed to CHX during the first 24 h, the formation of vinculin-positive adhesion plaques and intercalated discs was not altered even though the synthesis of new vinculin was inhibited  $\sim 95\%$ . If, however, CHX application was delayed for 24 h, then maturation of the intercalated disc failed to ensue and the fascia adherens junction retained immature structural properties and resembled the junction that developed after 24 h of exposure to ISO, alone (see Figs. 6 and 7).

## Discussion

Present observations demonstrate that contractile function



**Figure 13.** Relative turnover of MHC and vinculin in 12-d-old cultures exposed to ISO (●) or ISO + KCl (▽) for 48 h. ISO slows the rate of MHC degradation while accelerating vinculin breakdown when compared to control (○) cultures. ISO + KCl stimulates the degradation of both proteins. Each point is the mean  $[P/C]_t/[P/C]_0$  derived from five cultures.

and cell-cell contact act in concert to modulate the localization of the cytoskeletal-associated protein, vinculin, whose subcellular redistribution appears closely correlated with the maintenance of myofibrillar order. Adult heart cells disassemble and/or degrade elements of the contractile/cytoskeletal apparatus when cultured in a quiescent state. However, chronic exposure of these myocytes to  $\beta$ -adrenergic agonists or compounds that transiently elevate intracellular cAMP activate beating and promote the reassembly of myofibrils (Clark et al., 1991). In preparations where no cell-cell contact is apparent, nascent myofibrils become anchored to focal adhesions (Burridge et al., 1988) whereas in cultures where heart cells reassemble intercellular junctions, myo-

fibrils become preferentially linked to the fascia adherens (Geiger et al., 1990). Early phases of myofibrillogenesis (i.e., 1–24 h) do not require contractile/cytoskeletal protein synthesis nor are they accompanied by a sustained elevation in cAMP production; however, maturation of the intercalated disc during the second day of ISO treatment requires the synthesis of new cytoskeletal proteins. The results of these experiments suggest that: (a) nonbeating myocytes fail to form adherens-type junctions and disassemble myofibrils; (b) mechanical activity in the guise of rhythmic beating initiates formation of adherens junctions and myofibril reassembly; (c) beating-induced myofibrillogenesis uses a pool of pre-existing cytoskeletal proteins; (d)  $\beta$  agonist-induced cAMP production appears to regulate CS protein turnover but is not sufficient to initiate myofibrillar reassembly in the absence of beating; and (e) cell-cell contact modulates the subcellular site of myofibril reassembly.

Mechanical activity and neurohumoral factors are recognized as important modulators of contractile/cytoskeletal protein gene expression, turnover and myofibril assembly in cultured heart cells (Chien et al., 1991; Morgan and Baker, 1991; Simpson et al., 1991). Arresting the spontaneous contractile activity of neonatal heart cells by depolarizing the sarcolemma or blocking L-type  $Ca^{2+}$  channels depresses rRNA synthesis (McDermott et al., 1989), the expression of  $\beta$ -MHC transcripts, the synthesis and accumulation of MHC (Samarel and Englemann, 1991) and accelerates MHC proteolysis (Samarel et al., 1992). The consequence of this mechanical arrest is a rapid and progressive disruption of the myofibrillar apparatus that is reversible upon restoration of contractile activity (Samarel and Englemann, 1991). Nevertheless, it is also evident that  $\alpha$ - and  $\beta$ -adrenergic agonists regulate the expression of myofibrillar protein genes and promote the assembly of myofibrils independent of contractile activity (Chien et al., 1991; Simpson et al., 1991). Bishopric and Kedes (1991) further report that cell density can modify the response of neonatal heart cells to  $\alpha$ - and  $\beta$ -adrenergic agonists. In this latter investigation, skeletal  $\alpha$ -actin promoter transcription appears to be mediated by the  $\alpha$  adrenoceptor in cultures where cell-cell contact is minimized while the  $\beta$  receptor is the predominant stimulus of skeletal  $\alpha$  actin mRNA expression in cultures where the heart cells reestablish intercellular contacts. The observations summarized above imply that both protein kinase A

**Table III. Effects of Cycloheximide on Cytoskeletal Protein Turnover**

Protein	Treatment	Normalized P/C ratio <sup>§</sup>		
		Total protein	MHC	Vinculin
Synthesis*/Degradation <sup>†</sup>	$\pm$ ISO/CHX <sup>  </sup>			
	+ISO/–CHX	1.0	1.0	1.0
	+ISO/+CHX	0.09	0.06	0.04
	–ISO/+CHX	0.11	0.05	0.04
Degradation	+ISO/–CHX	0.84	0.84	0.72
	+ISO/+CHX	0.79	0.86	0.73
	–ISO/+CHX	0.82	0.78	0.74

\* Cultures were exposed to ISO for 48 h and labeled with  $[^3H]$ leucine during the last 4 h of ISO treatment. P/C ratio ( $^3H$  dpm/ $^{14}C$  dpm) was used to assess relative changes in protein synthesis.

<sup>†</sup> Double-labeled cultures were chased for 48 h in the presence or absence of ISO; changes in the P/C ratio reflect the degradation of nascent and long-lived proteins.

<sup>§</sup> Normalized P/C ratio =  $[^3H$  dpm/ $^{14}C$  dpm]<sub>48</sub> ÷  $[^3H$  dpm/ $^{14}C$  dpm]<sub>0</sub>; each value represents the mean normalized ratio derived from 9–12 cultures.

<sup>||</sup> CHX (100  $\mu$ g/ml) was added to culture medium from 24–48 h after ISO treatment.



(PKA) and protein kinase C (PKC) dependent pathways (see Morgan and Baker, 1991) are present and functional in cultured heart cells, and that cell density, mechanical activity and neurohumoral agents are capable of modulating cytoskeletal gene expression, protein turnover, and myofibrillar assembly, presumably, through these pathways. However, the regulation of cytoskeletal reorganization in relation to myofibrillogenesis in cultured heart cells remains to be resolved, and the present investigation focuses specifically on the turnover of some of the cytoskeletal proteins implicated in assembling and stabilizing myofibrils in muscle cells.

The experiments described in this investigation reveal that a significant depression in total and CS protein synthesis rapidly evolves in response to catecholamines (Figs. 10 and 11). This suppression of CS protein synthesis does not appear to impede myofibril reassembly over the first 24 h of ISO exposure, for myofibrillogenesis can proceed in the presence of sufficient CHX to block CS protein synthesis. Furthermore, cAMP production, the nominal second messenger in the PKA-dependent pathway (Gilman, 1987), is not sufficient to initiate CS reorganization in the presence of KCl, which effectively blocks ISO-induced beating even though the temporal kinetics of cAMP production (Table I) and CS protein synthesis parallel those observed in the absence of KCl (Fig. 11). Since the [<sup>3</sup>H]leucine content of actin, MHC and vinculin remains unchanged in the presence of ISO (Table I), these results suggest that the turnover of individual cytoskeletal proteins also must be regulated independently. Therefore, the rate of MHC degradation must be suppressed while that of vinculin must be enhanced to account for the static leucine content of each CS protein after 48 h exposure to ISO. An indirect measure of MHC and vinculin proteolysis confirms this hypothesis; furthermore, such experiments demonstrate that much of the newly synthesized CS protein (i.e., <sup>3</sup>H-labeled protein) is degraded over the first 48 h of ISO treatment (Figs. 12 and 13). These observations indicate that myofibril reassembly is driven, in part, by a posttranslational mechanism that appears to use a preexisting pool of CS proteins, presumably, derived from myofibrils disassembled and/or CS proteins synthesized but not assembled during the nonbeating state. Moreover, the CHX results also imply that this pool is sufficient to support the reorganization of the contractile apparatus during day 1 of ISO treatment; however, this pool appears to be depleted, thereafter, because CHX blocks further maturation of myofibrils during day 2—a period when all CS proteins except MHC display enhanced rates of synthesis (Fig. 11). Such experimental evidence supports the contention that the  $\beta$  agonist, ISO, modulates the turnover of CS proteins, but active work (i.e., beating) is required to facilitate the assembly of these proteins into myofibrils. The present observations also suggest that CS pool sizes vary considerably, because CS protein synthesis responds asynchronously to ISO with the P/C ratios rising within 12 h for actin,  $\alpha$ -actinin and desmin, followed by vinculin at 24 h, while MHC remains depressed even after 48 h of exposure to the catecholamine. These results further indicate that maturation of the intercalated disc appears to require the additional synthesis of vinculin and, perhaps, other CS proteins during the second day of exposure to catecholamine since CHX blocks remodeling of the junction, attachment of myofibrils to the intercalated disc (i.e., the fascia adherens) and the appearance of vinculin within Z-lines/costameres.

Unlike cultured embryonic (Sanger et al., 1984, 1986; Dlugosz et al., 1984; Lu et al., 1992) and neonatal (Atherton et al., 1986; Goncharova et al., 1992) heart cells that display spontaneous beating and assemble focal contacts, fascia adherens junctions and myofibrils, nonbeating adult heart cells disassemble their contractile elements (Decker et al., 1990, 1991; Clark et al., 1991), perhaps, because these quiescent myocytes are incapable of reassembling vinculin-positive focal contacts and/or intercellular junctions (Figs. 2 and 6) that are required to nucleate/stabilize myofibrils (Sanger et al., 1989; Goncharova et al., 1992; Lu et al., 1992). Although a direct role for vinculin in regulating myofibrillogenesis or stress fiber assembly is questionable based upon its in vitro affinity for the actin binding proteins (e.g.,  $\alpha$ -actinin) or “anchoring” proteins (e.g., talin or the catenins) (Burrige et al., 1988; Geiger et al., 1990), recent molecular/genetic investigations reveal that abnormal vinculin generated via transfection of cDNAs lacking the talin binding site (Bendori et al., 1989; Jones et al., 1989) or mutations in the vinculin, *deb*<sup>1</sup>, gene of *C. elegans* (Barstead and Waterston, 1991) promote the disruption of focal contacts, stress fibers and myofibrils. Proteolytic fragments of  $\alpha$ -actinin also are reported to induce adhesion plaque and stress fiber resorption in cultured fibroblasts (Pavalko and Burrige, 1991). These observations support the hypothesis that vinculin may assume a significant role in an in vivo environment in modulating stress fiber/myofibril integrity in a variety of cell types.

What signal transduction pathway(s) regulate(s) the redistribution of vinculin that develops in quiescent heart cells and what promotes its reincorporation into adhesion plaques and intercellular junctions upon exposure to ISO requires further investigation. Vinculin is reported to be phosphorylated in vivo on serine, threonine and tyrosine residues (Sefton et al., 1981), and recent experiments demonstrate that it can be phosphorylated by PKC which is localized in focal contacts (Werth et al., 1983; Jaken et al., 1989) and at other cytoskeletal sites (Mochly-Rosen et al., 1990). Nevertheless, agents which activate PKC (e.g., phorbol esters) are reported to dissociate vinculin from adhesion plaques and disassemble stress fibers and myofibrils (Burrige et al., 1988; Claycomb and Moses, 1988), making it unlikely that PKC-dependent phosphorylation of vinculin would account for its redistribution in the present investigation. Although the physical stretch associated with contraction could activate cardiac PKC (Komuro et al., 1991; Morgan and Baker, 1991), these observations also suggest that PKC-mediated phosphorylation of vinculin should dissociate this protein from adhesion plaques rather than facilitate its reassociation as is documented in the present study. The role of the PKA-dependent pathway also remains to be resolved, but present experiments suggest that an increase in cAMP and the concomitant activation of PKA that accompanies ISO exposure is not correlated directly with the incorporation of vinculin into junctions or adhesion plaques, rather it appears more closely associated with regulating the transcription/translation of cytoskeletal proteins. The present experiments strongly suggest that mechanical work modulates the distribution of vinculin and, perhaps, other cytoskeletal-associated proteins associated with myofibril assembly. What other second messenger(s) transduce the ISO-induced redistribution of vinculin and the reorganization of the contractile apparatus will be the focus of future studies.

The authors express their sincerest thanks to Monica Behnke-Barclay for preparing the micrographs and Laura Andersen and Donna Janes for isolating and culturing the feline myocytes.

This study was supported, in part from U.S. Public Health Service grant HL33616 and grants from NASA, the Chicago Metropolitan Chapter of the American Heart Association, and the Feinberg Cardiovascular Research Institute. D. G. Simpson was supported by a Markey fellowship in Cell, Molecular, and Structural Biology, Northwestern University; he is presently a postdoctoral fellow at the University of South Carolina Medical School, Columbia, South Carolina.

Received for publication 21 January 1993 and in revised form 14 June 1993.

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