

Pattern and Time Course of Rhodamine-Actin Incorporation in Cardiac Myocytes

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ABSTRACT Microinjection of skeletal actin labeled with rhodamine into cultured cardiac myocytes was followed by rapid incorporation of fluorescence into myofibrils of the cells. Myocytes examined as shortly as 5 min postinjection displayed fluorescent bands corresponding to the sarcomeres. By 10 min, distinct alternating wide and narrow bands of fluorescence were observed. The wide bands appeared to correspond to the full breadth of the I-bands, whereas the narrow bands of fluorescence corresponded to the M-lines. This pattern of fluorescence remained essentially unchanged for at least 15 h postinjection. The myofibrils of cardiac myocytes were functional after rhodamine-actin incorporation as judged by their ability to contract. The results of this study suggest that cardiac myofibrils are morphologically stable structures which, nonetheless, exhibit extensive exchange of actin subunits.

Myofibrils of embryonic cardiac myocytes and skeletal myotubes are morphologically stable structures with very similar ultrastructural features (1). Unlike skeletal muscle cells, however, myocytes are capable of DNA synthesis and mitosis after myofibril formation (2). The myocytes retain their myogenic state in culture for up to 6 wk (3), including their ability to contract spontaneously when sparsely plated, and to contract in coordination with other myocytes when in a high density monolayer (4).

Various studies have investigated the rates of myofibrillar protein synthesis and turnover in embryonic and adult myogenic tissues to elucidate the molecular state of myofibrillar metabolism. Incorporation of newly synthesized myofibrillar proteins into the contractile apparatus of embryonic cardiac muscle can occur within 2 h after incubation of the cells with a radioactive precursor (5) and without alteration of myofibril structure (6). Actin, a major constituent of the thin filaments, has a metabolic half-life of 12 d in adult heart tissue (7) and of 19 d in adult skeletal muscle (8). However, a study of actin turnover rates in cultured cells showed a half-life of 2.5–3 d in replicating mononucleated cells, and of 6 d in postmitotic myotubes (9), suggesting that embryonic tissues differ substantially from adult in protein turnover, or that *in vitro* conditions have stimulated metabolic activity.

This study was designed to investigate the dynamics of actin incorporation into myofibrils of embryonic cardiac myocytes grown in cell culture. Using the technique of fluorescent analog cytochemistry (10), microinjected rhodamine-actin was rapidly incorporated into myofibrils of these cells, which displayed

fluorescent bands that corresponded to the sarcomeres as early as 5 min postinjection. Maximum intensity and width of fluorescent bands occurred by 10 to 20 min postinjection. Incorporation of the fluorescent probe had no apparent effect on the ability of myofibrils to contract.

MATERIALS AND METHODS

Cell Culture Procedures: Hearts from 8-d chick embryos were minced in sterile magnesium- and calcium-free Saline G, and enzymatically dissociated for 15 min at 37°C. Digestions were terminated by addition of an equal volume F-12 nutrient medium containing 10% fetal calf serum and 30 µg/ml penicillin G and 50 µg/ml streptomycin sulfate. Cell suspensions were filtered through single layers of Nitex cloth, collected by centrifugation, resuspended in fresh nutrient medium and plated onto glass coverslips in 35-mm Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) at a density of $2-3 \times 10^5$ cells/dish. The dishes were kept in a humid atmosphere of 5% CO₂/95% air at 37°C. Microinjection was usually performed 24–48 h after plating.

Fluorescent Labeling of Actin: The actin labeling procedure and characterization of the fluorescent analog with regard to polymerization properties and presence of possible fluorescent contaminants have been described (Glacy, manuscript submitted for publication). Briefly, ~15 mg of skeletal muscle actin (11) was labeled with 10 mg of iodoacetamidotetramethyl rhodamine (Research Organics, Cleveland, OH), a sulfhydryl-specific dye which, based upon previous reports of sulfhydryl availability in actin (12, 13), should react preferentially with cysteine 373. Rhodamine-actin preparations were purified of free dye, denatured actin, and trace contaminants by repeated cycles of polymerization/depolymerization and Sephadex G-25 gel filtration.

Microinjection and Microscopy: Microinjection of cardiac myocytes was performed according to standard procedures (Glacy, manuscript submitted for publication). A Zeiss Standard microscope, mounted on a Leitz baseplate, was used for microinjection, and both phase-contrast and fluorescence

microscopy. Fluorescence exposures were ~30–40 s using Ilford XP-1 35mm film developed for 6.5 min at 40°C in Ilford XP-1 chemicals.

RESULTS

Cardiac myocytes in vitro were assigned two general morphologies: elongated cells with numerous side processes, which frequently made contact with other cells; and myocytes with a fibroblastic morphology, which were difficult to distinguish from nonmyogenic cells in the cultures. Both types contained numerous cross-striated myofibrils, contracted in a rhythmic and spontaneous fashion, and appeared to incorporate rhodamine-actin identically. As with fibroblastic cells (Glacy, manuscript submitted for publication), living cardiac myocytes observed 1 h after microinjection of FITC-ovalbumin displayed only non-specific cytoplasmic fluorescence.

Both beating and quiescent myocytes were microinjected. In the case of beating cells, microinjection either temporarily halted the activity or had no effect. Penetration of the cell membrane of nonbeating cells by the micropipette usually was followed by a peristaltic-like wave of contraction that moved down the cell. Glycogen deposits, typically found in cultured myocytes (reference 3, and Fig. 1c), were dispersed when directly in the path of injected actin solutions. Microinjection had no apparent effect on the structure of existing myofibrils. Since myofibrils could be seen by phase-contrast microscopy during initial stages of fluorescent actin incorporation, it is unlikely that development of the fluorescent patterns in these cells represented construction of myofibrils rather than utilization of rhodamine-actin by existing structures.

The pattern of rhodamine-actin incorporation into myofibrillar structures immediately following microinjection usually was difficult to discern, due to initially uniform cytoplasmic fluorescence. By 5 min postinjection, however, fluorescent bands periodically distributed along the myofibrils were visible. By 10 min, distinct alternating bands of fluorescence were seen, particularly in thinner peripheral cell areas where the fluorescence pathlength was shortest and cellular structures least obscured by diffuse cytoplasmic fluorescence contributed by any unincorporated rhodamine-actin (Fig. 1a and b).

Fig. 1c and d represent an injected myocyte at 1 h postinjection. Exceptionally clear fluorescent patterns are evident in this cell. Comparison of these patterns to those seen in isolated skeletal myofibrils stained with antiactin antibodies revealed an identical fluorescence distribution, indicating that the wide fluorescent bands in the microinjected cells corresponded to actin-containing I-bands (14). Additionally, high magnification of fluorescent myofibrils revealed that the wide fluorescent I-band is bisected by a slightly darker zone, most likely representing the Z-line (Fig. 1d, inset). Interestingly, microinjected cardiac myocytes contained thin lines of fluorescence which alternated with the I-bands. These narrow fluorescent bands corresponded to the M-line region, an area presumably free of F-actin. Previous immunofluorescence studies of skeletal myofibrils stained with antiactin antibodies also have resulted in fluorescent M-lines (14, 15). Gel electrophoresis of rhodamine-actin indicated fluorescence only at the 43,000-mol wt position, thus making it unlikely that the thin fluorescent M-lines in microinjected myocytes represented incorporation of a trace contaminant, unless that contaminant was of identical molecular weight to actin. Furthermore, incubation of permeabilized, fixed myocytes with rhodamine-actin showed no discernible myofibrillar fluorescence, therefore providing evidence against

binding of rhodamine-actin to the M-line. Thus far, it cannot be ruled out that the M-line fluorescence represents a type of rhodamine-actin utilization by the myofibrils.

At 2 h postinjection, fluorescent patterns remained essentially unchanged, still demonstrating alternating wide and narrow bands of fluorescence (Fig. 1e and f). Observation of microinjected myocytes up to 15 h postinjection indicated no further qualitative changes in fluorescence distribution. Myocytes at all stages of observation following microinjection retained the ability to contract.

Nuclei of most microinjected cells excluded the injected probe. The slight fluorescence seen in the nuclei of some injected myocytes may actually represent perinuclear fluorescence rather than intranuclear rhodamine-actin (Fig. 1d and f). Additionally, not all microinjected rhodamine-actin was incorporated into myofibrils. Rhodamine-actin not associated with sarcomeres maintained a diffuse cytoplasmic distribution that appeared to correspond to cell thickness (Fig. 1f). This unincorporated probe may have become part of the cell's pool of nonfilamentous actin.

DISCUSSION

This study demonstrates that rhodamine-actin, microinjected into cultured cardiac myocytes, was rapidly incorporated into myofibrils of the cells. Maximum intensity and width of fluorescent banding patterns in microinjected myocytes were achieved by 10–20 min post-injection, indicating that a highly dynamic relationship exists between actin of the thin filaments and cellular nonmyofibrillar actin. Fluorescent patterns of rhodamine-actin incorporation in living cells were identical to those previously obtained by antiactin antibody staining of isolated myofibrils (14, 15).

Incorporation of rhodamine-actin into myofibrils and its apparent lack of effect on the ability of microinjected cells to contract, suggest that modification of actin with rhodamine did not impair its physiological properties nor the cells' ability to recognize it. Additionally, unincorporated rhodamine-actin remained diffuse, rather than polymerizing as the general intracellular ionic conditions would favor, indicating integration of the probe into the cells' functional actin pool.

Although rhodamine-actin is incorporated rapidly into sarcomeres, the time course of that incorporation reveals little concerning the detailed steps of the process. If rhodamine-actin served only as a tracer in endogenous actin pools, and not as a perturbant of cellular functions, then there are at least two possible mechanisms by which rhodamine-actin may integrate into myofibrils. The first possibility is that incorporation of rhodamine-actin into myofibrils is simultaneous with removal and degradative turnover of endogenous myofibrillar actin. However, biochemical evidence suggests a half-life for actin of 6 d in cultured myotubes (9), a period that appears incompatible with the rapid time course seen in the present study. A second possibility is that actin of the thin filaments may be in a continuous state of exchange with nonmyofibrillar actin. This would result in a cycling of cellular actin through sarcomeres, accompanied by little protein degradation. The results of this study suggest that this exchange is extremely rapid; by 10–20 min postinjection, sarcomeres appear fluorescent to the full width of the I-bands. Furthermore, sarcomeres remain fluorescent for at least 15 h following injection. These facts suggest a mechanism whereby immediately following microinjection, the molecular exchange between thin filaments and nonmyofi-

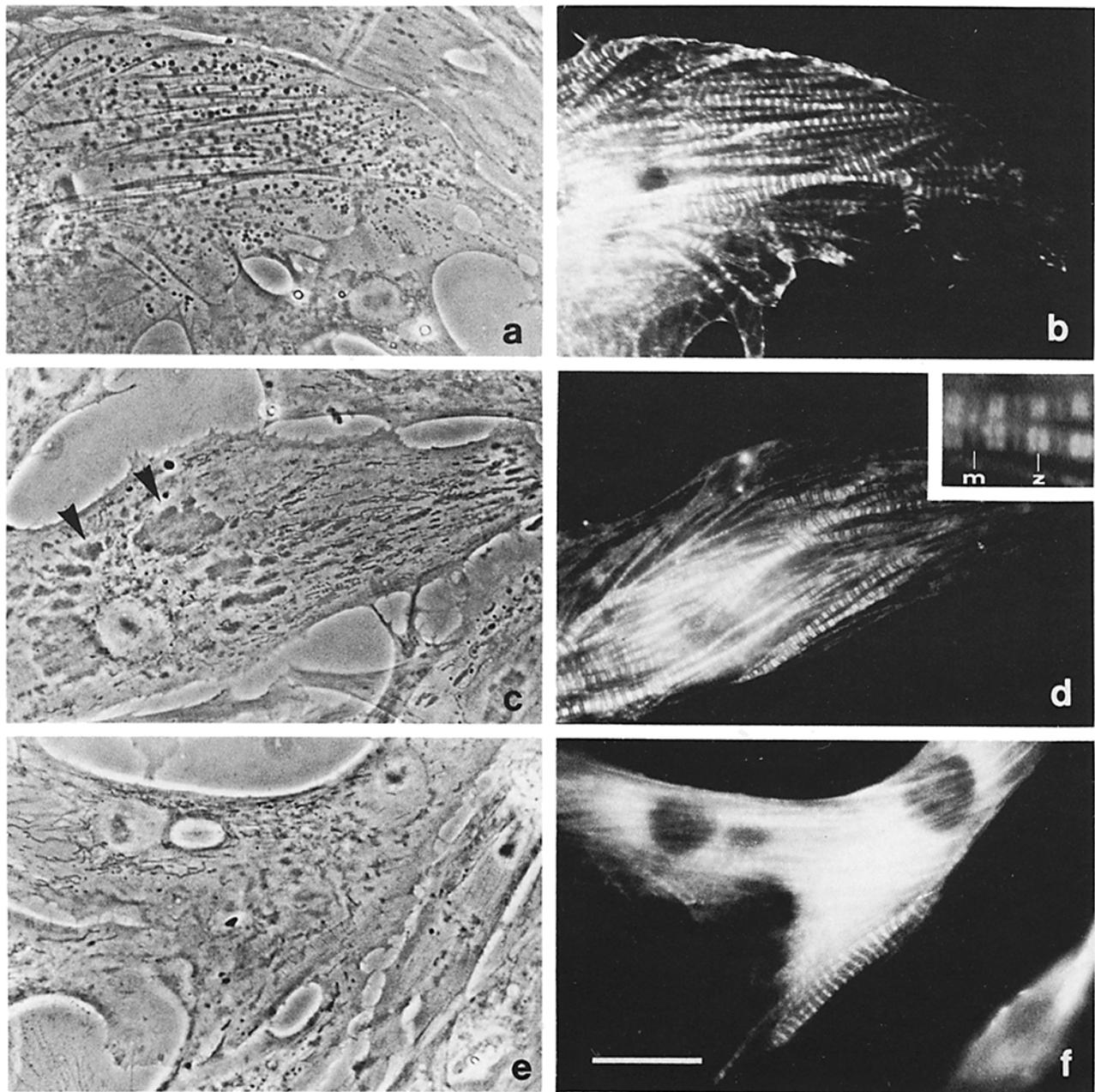


FIGURE 1 Phase-contrast and corresponding fluorescent images of embryonic cardiac myocytes microinjected with rhodamine-actin. Time points following injection are 10 min (a and b), 1 h (c and d), and 2 h (e and f). Cells at all three stages show rhodamine-actin incorporation. The inset of d is a high magnification micrograph of fluorescent myofibrils demonstrating the slightly darker Z-line and fluorescence in the M-line. Glycogen deposits, typically found in cultured myocytes, are indicated by arrowheads in c. Bar, 20 μ m; \times 840. Inset, \times 3,400.

brillar actin would be represented predominately by incorporation of rhodamine-actin molecules and removal of unlabeled endogenous actin molecules. Eventually, a balance would be achieved in which numbers of rhodamine-actin molecules entering and leaving the sarcomeres would be approximately equal. This proposal would account for the rapid appearance of fluorescence in the myofibrils and its subsequent persistence.

Additionally, the likelihood that the time course of incorporation is artifactual must be considered. Injection of rhodamine-actin may upset the intracellular equilibrium between actin synthesis and utilization by abruptly presenting the cell with an expanded pool of actin. In this case, the cell may very

well respond by increasing myofibrillar protein turnover, with concomitant incorporation of rhodamine-actin from the new pool.

This microinjection study has demonstrated that myofibrils, heretofore considered static cytoplasmic structures, exhibit a dynamic molecular relationship with nonmyofibrillar actin. Presuming actin monomer addition solely at the ends of the thin filaments, the presence of fully fluorescent I-bands by 10–20 min after injection implies that at least some thin filaments are completely turned over once every 10–20 min. However, more complex processes for subunit exchange may exist and possibilities such as monomer exchange along the

length of thin filaments or incorporation of actin as oligomers or short fragments cannot be eliminated (16). Additionally, fluctuations in local cellular ionic concentrations may have a significant effect on actin subunit exchange (17). Our understanding of this process is still rudimentary.

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