DEOXYRIBONUCLEIC ACID SYNTHESIS AND MITOSIS IN DIFFERENTIATED CARDIAC MUSCLE CELLS OF CHICK EMBRYOS

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INTRODUCTION

Within the past decade, numerous investigators have contended that neither DNA synthesis nor mitosis can occur in cells which have synthesized specialized or so-called "luxury" proteins (1–8). In particular, the idea has been widely promulgated that once a muscle cell has synthesized myofilaments, it is no longer capable of replicating its DNA or undergoing mitosis. Stockdale and Holtzer, for example, working on 10 day old chick embryonic thigh muscle in vitro and 3–4-day old chick somites in vivo, concluded that myoblasts which are synthesizing contractile proteins do not synthesize DNA and do not divide (1). Okazaki and Holtzer (2, 3) stated that nuclei regulating the synthesis of myosin cannot replicate DNA. Radioautographic studies indicated that the decision to translate for myosin and to withdraw from the cell cycle was acted on in the same G_1 in a myoblast that synthesized DNA for the last time about 8 hr previously (4–6). Coleman and Coleman (7) also observed that presumptive skeletal muscle cells which incorporate thymidine-^{3}H do not bind fluorescein-labeled antibody to myosin.

All of the studies which form the basis of these generalizations about myofibril differentiation (1–7) have been done with skeletal muscle. Yet, it is difficult here to separate cell fusion, ending of DNA synthesis, and initiation of myosin synthesis because the three processes occur concurrently. In cardiac muscle, however, the differentiating cells do not fuse. Thus, by studying cardiac muscle, we thought that we could judge whether an obligatory relationship exists between cessation of DNA synthesis and onset of myosin synthesis, or whether it is cell fusion in skeletal muscle that turns off DNA synthesis. Shafiq et al., in an electron microscope study of skeletal muscle and cardiac muscle from 3-wk old rats, reported that no mitotic figures were found in either the skeletal muscle fibers or in myocardial cells containing myofibrils (8). Manasek, however, in the same year, reported mitotic figures in electron micrographs of chick cardiac muscle cells that already contained myofibrils (9). Using cinematography, Mark and Strasser (10) noted

that cardiac muscle cells from 1- to 4-day old rats continued to beat while dividing in vitro. These investigators observed a mitotic cell that contained striated myofibrils. DeHaan (11) also concluded that myocardial cells proliferating in vitro are partially differentiated. Other light microscopists have noted that cardiac muscle expands as a unit tissue instead of recruiting new cells from an undifferentiated precursor pool (12–15), and Polinger (16) reported that cardiac muscle cells containing glycogen can incorporate thymidine-^{3}H.

This paper confirms Manasek's electron microscopic observations of mitoses in cardiac muscle cells that contain myofilaments (9) and records the first demonstration by electron microscopic radioautography of myofilaments in the cytoplasm of myocardial cells in the S phase of the cell cycle. In the stage 24–26 embryos studied, all cardiac muscle cells contain myofibrils. When the plane of section is favorable, Z bands can be identified in addition to A and I bands within the myofibrils of muscle cells that incorporate thymidine-^{3}H.

MATERIALS AND METHODS

The ventricles of chick embryos in Hamburger-Hamilton stages 24–26 (4–5 days) were studied because previous work (12) indicated that the ventricle exhibits a high mitotic rate during this period. The embryos were removed from the yolk and placed into either Hanks' balanced salt solution or into L-15 tissue culture medium. The hearts were excised while still beating and either fixed immediately or incubated in 10 μCi of thymidine-^{3}H (New England Nuclear Corp., Boston, Mass.) in 0.5 ml of L-15 for 1 hr at 37°C. The hearts appeared healthy and continued to beat during the incubation.

Fixation was carried out in Karnovsky's formaldehyde—glutaraldehyde fixative at room temperature, followed by osmium tetroxide. While in the first fixative, the hearts were cut into small blocks. These were washed in several changes of cacodylate buffer (pH 7.4) for approximately 15 min before they were immersed in a cold 2% solution of osmium tetroxide in s-collidine buffer for 1 hr. After the second fixation, the tissues were washed in maleate buffer (pH 5.2) for 15–30 min and then stained en bloc in a 2% solution of uranyl acetate in maleate buffer (pH 6.0). Dehydration through a graded series of alcohols and clearing in propylene oxide followed. Finally, the tissues were embedded in Araldite. Hearts were also fixed in Bouin's fluid 1 and 4 hr after immersing in thymidine-^{3}H. These were embedded whole in paraffin for serial sectioning.

For electron microscopy, thin sections of Araldite-embedded tissue were supported on uncoated copper grids. Thicker sections of Araldite- and of paraffin-embedded material were placed onto specially cleaned gelatin-coated slides for radioautography at the light microscope level. In experiments involving light microscopic radioautography, the slides were dipped into Ilford K-5 photographic emulsion (Ilford Ltd., Ilford, Essex, England) in the darkroom and developed in Dektol (Eastman Kodak Co., Rochester, N. Y.) 1, 2, and 3 wk later. They were stained with a solution of 1% toluidine blue in 0.1% borax. For electron microscopic radioautography, grids were placed on a gelatin-coated slide in the darkroom and a film of Ilford L-4 emulsion was placed over the grids by using a chromium wire loop. After a period of 3 wk, the grids were developed in filtered D-19 and stained for 5 min in 0.2% lead citrate.

RESULTS

Mitoses in partially differentiated myocardial cells containing myofilaments were observed in the chick embryos at stages 24–26. These mitotic muscle cells have been well illustrated by Manasek (9) in 7–11-day chick embryos and will not be illustrated again here. The principal feature of interest in the dividing cells is the presence of thick and thin filaments arranged for the most part in thin myofibrils. Like Manasek, we failed to observe Z bands in dividing cells. The mitotic index in the ventricle at this time is between 2 and 3%, and thus the total number of mitoses that one observes is not very high. Since contracting myocardial cells can divide in vitro (10), our failure to observe Z bands in mitotic cells is probably due to our small sample rather than to transient loss of myofibril organization during division. Most of the sections are oblique to the long plane of the myofibrils because cardiac muscle cells course in irregular bundles that alternate in direction from one cell layer to the next in the heart wall.

Light microscopic radioautography of cells fixed 1 hr after administration of thymidine-^{3}H reveals that about 20% of the cardiac cells are in DNA synthesis at any one time (14–16). The labeled cells are uniformly distributed throughout the outer myocardium. All of these labeled myocardial cells, when examined by electron microscopy, proved to contain myofibrils. As we noted above, thin sections of the intact cardiac wall seldom show myofibrils cut longitudinally over a significant portion of their length (Figs. 1, 2). One can identify thick (myosin) and thin (actin) fila-

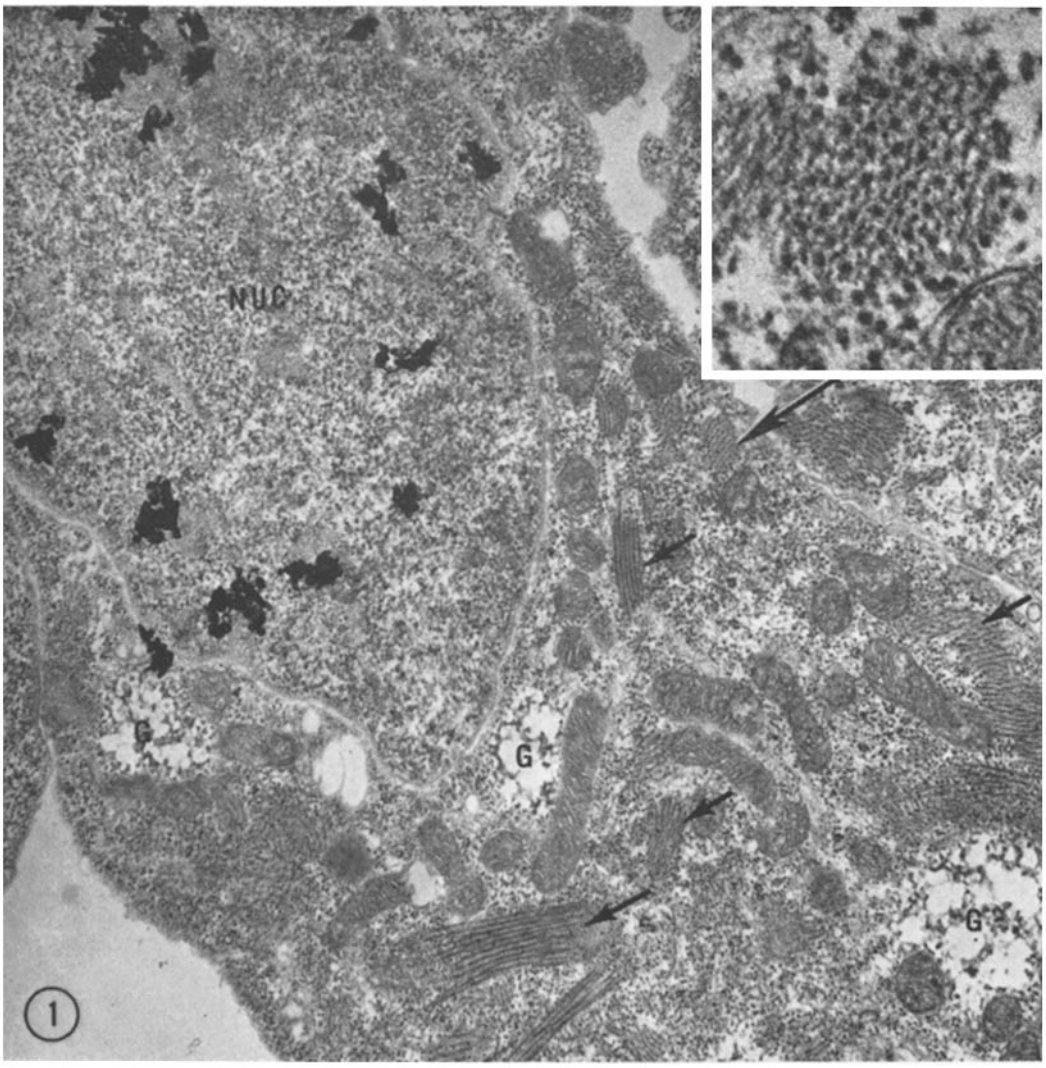

FIGURE 1 Electron micrograph of a radioautograph of a cardiac muscle cell fixed after 1 hr of continuous exposure to thymidine-^{3}H. Numerous silver grains can be seen over the nucleus (*NUC*), indicating the presence of radioactive DNA synthesized by this partially differentiated cell. Arrows call attention to myofibrils in various planes of section. The *inset* shows at higher magnification a cross-sectioned myofibril like that at the large arrow. The empty-appearing areas (*G*) in the main figure are partially extracted glycogen deposits. × 20,000; *inset*, × 100,000.

ments, A and I bands in all the labeled cells, whatever their orientation, and in the rare longitudinal section Z bands can be detected (arrows, Fig. 3). In cross-section (*inset*, Fig. 1), the typical arrangement of the interdigitating sets of thick and thin filaments is seen within the myofibril.

The mononucleated myocardial cells are attached by desmosomes and by primitive intercalated discs into which the myofibrils insert (Fig. 3). Mitochondria and free ribosomes are numerous (Figs. 1, 2). The glycogen deposits that characterize the cardiac muscle cells (16–18) are partially extracted (Fig. 1) due to treatment of the tissue with uranyl acetate en bloc. Each myocardial cell has a prominent Golgi apparatus (Fig. 2) which may be involved in the synthesis

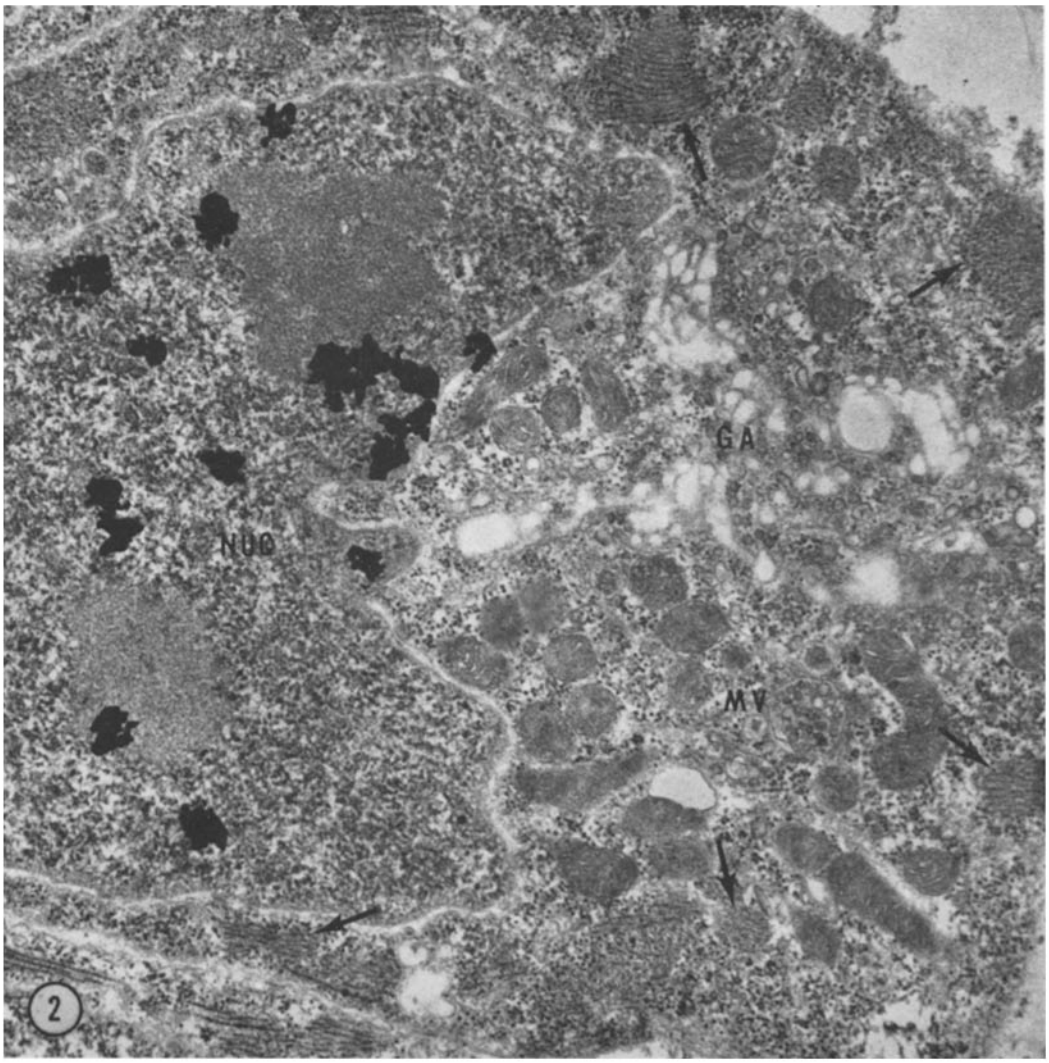

FIGURE 2 Electron micrograph of a radioautograph of a cardiac muscle cell exposed to thymidine-^{3}H for 1 hr. The numerous silver grains indicate that the nucleus (*NUC*) was synthesizing DNA prior to fixation. This particular section shows that the myocardial cell contains a well-developed Golgi apparatus (*GA*), in addition to myofibrils (arrows), mitochondria, and ribosomes. A multivesicular body (*MV*) is associated with the Golgi apparatus. This growing contractile cell is probably also engaged in the synthesis and secretion of chondroitin sulfate or other secretory products. × 25,000.

of sulfate-rich cardiac jelly (19, 20). We also observed the dense granules (*GR*, Fig. 3) in the Golgi complexes noted by Manasek (18) in 4–5-day old heart muscle cells.

The myocardium still retains its originally epithelioid nature as part of the splanchnic mesoderm, and the endothelial cells in the walls of newly invading vessels are essentially the only other cell type present at this time. The latter often contain labeled nuclei. Rare mesenchymal cells and no "undifferentiated" cells ("myoblasts") are seen in the 4–5 day myocardium.

DISCUSSION

All the myocardial cells in the 4–5 day chick heart which label with thymidine-^{3}H after 1 hr of

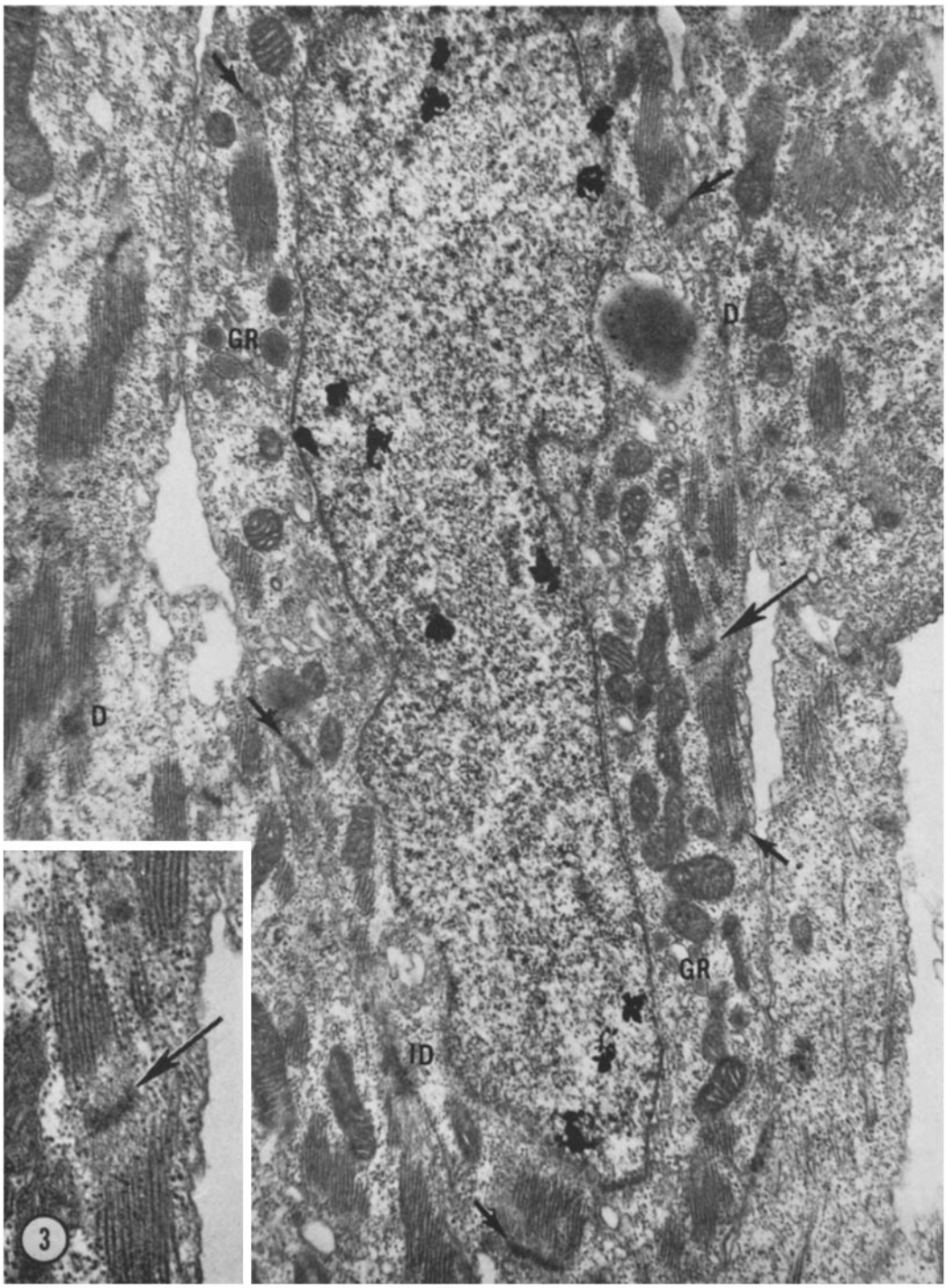

FIGURE 3 Electron micrograph of a radioautograph of a cardiac muscle cell exposed to thymidine-^{3}H for 1 hr. In this section, myofibrils are cut nearly in the longitudinal plane. In addition to A and I bands, it can be seen that Z bands are present (arrows). The *inset* shows at higher magnification the myofibril labeled by the large arrow. In the main figure, desmosomes (*D*) and a primitive intercalated disc (*ID*) can be seen connecting the growing cells. Dense granules (*GR*) within the Golgi complex can also be identified. × 20,000; *inset*, × 50,000.

exposure to the radioactive DNA-precursor contain myofibrils. Thus, unlike the situation in developing skeletal muscle (21), there seems to be no pool of undifferentiated myoblasts in the developing heart. In cardiac muscle, it is the differentiating cells that are dividing, and they continue to do so for many generations. While it could be argued that the myocardial cells do not synthesize muscle proteins and DNA at the same time, the cell doubling time is so short that this possibility seems highly improbable (15). Double isotope experiments might help to settle this point, but would be less convincing for myosin than for chondroitin sulfate (22) or collagen (23), because no specific myosin precursor is available. In this context, it is noteworthy that cardiac muscle cells are probably also producing chondroitin sulfate (19, 20). Thus, it is not unlikely that this differentiating cell type is simultaneously synthesizing DNA, myofibrils, and connective tissue ground substance.

The fact that skeletal muscle cells, unlike cardiac muscle cells, do not synthesize DNA after they begin to make myofibrils could be explained, as we indicated in the introduction, by the fact that skeletal muscle is syncytial. Indeed, Okazaki and Holtzer have suggested that the shut-off mechanism for DNA synthesis has to go into effect before myoblast fusion can take place (2, 3). Cell fusion seemingly does not occur until the cell has entered the G_1 following a terminal S phase. The same sequence requiring a terminal G_1 is said to hold for myofibril synthesis in skeletal muscle (4–6), but, as we have seen here, cannot be true for cardiac muscle. It is tempting to speculate that in skeletal muscle, a control mechanism is introduced which delays the onset of myofibril synthesis until *after* cell fusion. Such a mechanism would account for the apparent relation between the onset of myofibrillogenesis and a terminal mitosis, when in fact myofibrillogenesis per se is compatible with continued cell proliferation. Against this hypothesis is the light microscopic observation of fluorescein-labeled antimyosin over so-called mononucleated, elongated somite cells in vivo and observations of antibody-labeled myosin in mononucleated muscle cells in vitro at 24 hr (2, 3). The latter could be explained by trypsin fragmentation of myofibers (2), rather than by synthesis of new muscle proteins in mononucleated myoblasts, for myofibril synthesis probably begins later in such cultures (7). The idea that cell fusion results in cessation of DNA synthesis holds for other naturally occurring syncytia, such as osteoclasts and the trophoblast (24). An apparent exception is the fact that cells induced to fuse by Sendai virus may continue to make DNA if conditions are appropriate (25). Moreover, other viruses induce multinucleated muscle fibers to synthesize DNA (26, 27). It would be interesting, however, to explore further the normal interrelations between cell fusion, DNA synthesis, and cell differentiation.

Finally, we should emphasize that the DNA synthesis demonstrated in cardiac muscle cells during embryogenesis is related to cell division and is not associated with any significant endoreplication of DNA. Mitoses are common (9–12) and the diploid amount of DNA seems to be present in each cardiac cell nucleus (13). It cannot be argued that "dividing cardiac myoblasts degenerate, become polyploid, or form binucleate cells," or that "differences between cardiac and skeletal muscle cells are minor variations on the same theme" (28, page 552). Rather, the time has come to take a critical look at the quantal mitosis theory of differentiation as applied to muscle.

SUMMARY

This electron microscopic study of radioautographs has shown that myocardial cells from chick embryos at stages 24–26 are capable of actively synthesizing DNA, although the cells are in a differentiated state as judged by the presence of actin and myosin filaments associated in typical myofibrils with A, I, and Z bands. Oft repeated statements that differentiated cells are not capable of dividing clearly do not apply to cardiac muscle.

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