MICROTUBULES IN MAMMALIAN HEART MUSCLE

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ABSTRACT

Sections of adult mammalian cardiac muscles fixed at room temperature reveal numerous microtubules (24–28 nm in diameter) both near the nucleus and in the extra-myofibrillar space. Microtubules encircle the nucleus, are associated with the myofibrils in a helical arrangement, and form a network that runs transversely at the level of the I band and axially between the myofibrils. Microtubules are more numerous in muscle cells than previously recognized and may perform more than a cytoskeletal function.

KEY WORDS microtubules · adult muscle · ultrastructure · mammalian · cardiac

Microtubules have been described in a wide variety of eukaryotic cells (for reviews, see references 10, 21, and 27), but have been mentioned rarely in studies of cardiac fine structure (6, 22, 26) until recently (25). Microtubules are hollow cylinders with an average outer diameter of 24 nm and an average inner diameter of 12 nm when viewed in thin sections after conventional staining procedures with uranyl acetate and lead citrate. The wall is composed primarily of heterodimers of tubulin (2, 5) which can be seen in cross section most often as a ring of 13 subunits of 5 nm diameter. A microtubule network has been observed in a variety of intact cells (24) and in cells in tissue culture (1, 31), along with a network of stress fibers—actin filament bundles (8, 9, 16). Groups of microtubules are also found in association with 10-nm filaments (8).

Microtubules have been described in developing muscle (7, 30) and in adult skeletal muscle (29). Tubulin has been synthesized on polysomes from embryonic chick skeletal muscle (14). Although a cytoskeleton of microtubules has been reported in many cells of asymmetric shape, a microtubule network in adult muscle has not been described. In this communication we report the distribution of microtubules in mammalian heart and discuss several possible functions for these microtubules. Microtubules encircle the nucleus, are associated with the myofibrils in a helical arrangement, and form a network that runs transversely at the level of the I band and axially between the myofibrils. A brief report of some of these data has been presented (12).

MATERIALS AND METHODS

Strips of anterior and posterior papillary muscles of seven adult dog hearts and three cat hearts were held at rest length during fixation at room temperature with 4% paraformaldehyde-5% glutaraldehyde in Millonig's phosphate buffer at pH 7.3 for 1 h. Whole papillary muscles from seven guinea pig hearts and three rat hearts were fixed in situ. Muscles were then cut into 1-mm cubes and fixed for an additional 2 h.

For comparison, several hearts were fixed in 5% glutaraldehyde in a 100 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]) buffer containing 2 mM EGTA (ethylene glycol-bis[β-aminoethyl ether]N,N'-tetraacetic acid), 1 mM MgSO₄, 1 mM GTP at pH 6.9, a medium which has been shown to provide increased visualization of microtubules (17). In these experiments, one guinea pig heart was fixed at 37°C and one at 25°C. Three dog hearts were fixed at 25°C, and one dog heart was fixed at 0°C. Still another dog heart was fixed at

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acetate followed by lead citrate, and were examined in flat silastic molds. Thin sections were cut with a diamond knife, stained with uranyl acetate or uranyl magnesium acetate followed by lead citrate, and were examined in an RCA EMU4 or a Philips 201 electron microscope.

Profile lengths were measured with dial calipers in prints (total magnification $\approx 30,000$) of longitudinal sections from five different dogs. The numbers of cross-sectional microtubule profiles were counted in prints (total magnification $\approx 40,000$) of cross sections, and each average number was graphed as a ratio for each group. The mean, standard error of the mean (SEM) and standard deviation were computed for each set of measurements. The number of measurements for each group was set by monitoring the size of the SEM and the animal-to-animal variation. For example, the number of measurements in longitudinal sections for each dog was 60 and for each guinea pig was 50. The number of measurements in cross sections for all animals was at least 25, and for dog no. 1 the number of measurements for this papillary muscle was 98.

RESULTS

Microtubules were routinely visible in our preparations of cardiac muscle cells fixed at room temperature in phosphate buffer. Microtubules were seen near the nucleus and in the myofibril-free areas throughout the cell from nucleus to cell surface. Heart cells fixed in a polymerization medium (PM) at room temperature showed longitudinal profiles $\geq 2 \mu m$ in length, clear profiles of centrioles near the nucleus or near the Golgi saccules in sections not containing a visible nucleus, well-preserved thin filaments and 10-nm filaments in the I band, and dense tufts on the T-tubules and beneath the sarcolemma. Microtubules were preserved in PM at 37° and 0°C, but fixation at 25°C gave the best overall cell morphology. Tannic acid in the fixative gave additional density to the outer wall of the microtubule.

Electron micrographs from four dog hearts and three guinea pig hearts fixed at room temperature were used for measurement of average diameter, wall thickness, and length. In cross sections of microtubules, the outer diameter was $26.2 \pm 0.2 \text{ nm} (n = 108)$ and the inner diameter was $10.7 \pm 0.2 \text{ nm} (n = 86) \text{ (SEM)}$, which gives an average wall thickness of $7.7 \text{ nm}$. Fig. 1 shows a microtubule $24 \text{ nm}$ in diameter with a wall thickness of $6 \text{ nm}$ and three clear subunit profiles. Markham rotation enhanced the subunit structure at $n = 13$ when compared to $n = 12$, $n = 14$, and $n = 15$. Too few of the subunits were sufficiently clear to give a useful diffraction pattern. Very few of the cross-sectional profiles of microtubules in cross sections of muscle were exactly normal to the plane of section, and subunits were difficult to identify.

In longitudinal sections of muscle, the length of the microtubules observed near the nucleus depended on the shape of the nucleus. The longest profile observed ($3.5 \mu m$) was next to a smooth-surfaced nucleus and oriented parallel to the myofibril axis (see Fig. 2a). The shortest profiles were observed near the convoluted nuclei (see Fig. 2b). In the myofibril-free areas of the muscle cell in longitudinal sections, the microtubules varied in length from 0.3 to $2.9 \mu m$. The longest profiles were oriented along the myofibril axis.

There was no significant difference between the average length of microtubule profiles in longitudinal sections of dog heart fixed in phosphate-buffered aldehyde ($I$) compared to that in glutaraldehyde in PM ($II$) (Fig. 3a). Similar values for the average length of microtubule profiles were measured in guinea pig hearts (Fig. 3a). The number of cross-sectional microtubule profiles in cross sections of cardiac muscle were also measured in groups I and II. Again, there was no significant difference between the number of profiles per unit cross-sectional area of muscle in the two fixation conditions, both in dog hearts and in guinea pig hearts (Fig. 3b).

In cross sections of heart muscle, microtubules were seen near the nucleus and in myofibril-free areas throughout the cell. Cross-sectional profiles spaced around the nucleus at irregular intervals from 0.5 to $2.0 \mu m$ predominated when the nucleus had a smooth round shape. Longitudinal profiles of random orientation were seen when the nucleus was quite lobular. Cross-sectional profiles of microtubules were seen at all levels of the myofibrils. An occasional microtubule was seen in the middle of a myofilament bundle (Fig. 4a), but most were located at the edge of the myofilament bundles, often near a mitochondrion (Fig. 4b and c). Longitudinal profiles were seen near the Z bands in cross sections (Fig. 4d) and rarely near the M band.
In longitudinal sections of heart muscle, the cross-sectional profiles of microtubules were located at the edge of the I band near the Z band (Figs. 2b, 5a), less frequently at the M band, and seldom at the A-band level. They occurred most often singly, but sometimes two or three were grouped in a 150-nm area. Longitudinal profiles were always seen in sections which grazed the surface of the myofilament bundles and included extended regions of the sarcoplasmic reticulum (SR) network (Fig. 5b). Such profiles were also seen between myofilament bundles when portions of the SR or mitochondria were viewed (Fig. 5a).

The distribution of microtubules in longitudinal sections was consistent with the distribution in cross section. A longitudinal section of dog cardiac muscle fixed in phosphate-buffered paraformaldehyde-glutaraldehyde at room temperature shows the typical arrangement of microtubules (Fig. 6). Comparable sections of hypertrophied guinea pig heart muscle showed similar profiles of microtubules. Longitudinal sections of dog cardiac muscle fixed in PM with glutaraldehyde at room temperature also showed similar profiles (Fig. 7). Figs. 6 and 7 show microtubule profiles with a sigmoid shape in sarcomeres at or near rest length. No longitudinal profiles were seen between the myofibrils in longitudinal sections when the SR and T-system profiles were very scant and mitochondria were not visible.

Fig. 8 is an artist's sketch of a longitudinal section which illustrates the different microtubule profiles observed in adult heart muscle. Some microtubules run parallel to the myofibril axis. In favorable sections, however, a slight sigmoidal shape is detected in these microtubules, and they often extend across the Z band to a second sarcomere (see Figs. 5a and 7a). Microtubules run transverse to the myofibril axis at the level of the I band—some for only ~1 μm before they appear to curve out of the plane of section, and some for >1 μm. The transverse profile at the I band is often continuous with a longitudinal profile along the fiber axis between two myofibril bundles (see Fig. 7f). The large majority of microtubule profiles curve diagonally across the sarcomere in the plane which contains the sarcoplasmic reticulum between the myofibril and the mitochondria. Some of these microtubules extend to a second sarcomere (see Fig. 7e). Occasionally, two microtubule profiles approach the middle of the sarcomere from different directions.
Figure 2  (a) Straight longitudinal profiles of microtubules (arrows) next to a smooth-shaped nucleus in a shortened muscle (sarcomere length = 1.7 µm). × 56,000.  (b) Random profiles and short segments of microtubules (arrows) seen in region near lobulated nucleus. Note sarcomere size is similar to that previously shown in Fig. 2a (1.5 µm). Both electron micrographs are taken from hearts fixed in PM at room temperature. Microtubule in cross section near I band is shown at arrowhead. Bar, 1 µm. × 56,000.
thus suggesting an opposing double-helical array. Sometimes two microtubules in adjacent sarcomeres of the same myofibril have the same sigmoidal shape (see Fig. 7g). All of these profiles suggest that the microtubules wrap around the myofilament bundles.

The data from cross sections support the contention that the microtubules coil in a helical fashion but with different pitches. The longitudinal profiles of microtubules in cross sections of muscle are at the I band only, are less than 1 μm, and follow the contour of the myofilament bundle. Cross-sectional profiles are unevenly distributed between the myofibrils and they are seldom

\[ \text{FIGURE 3} \ (a) \text{ Average length of microtubule profiles (in μm) in longitudinal sections of papillary muscles from five dog hearts and five guinea pig hearts is charted, including each standard error of the mean. Hatched bars represent paired samples from a single dog. Animals in group I are fixed in Millonig’s phosphate-buffered paraformaldehyde-glutaraldehyde. Animals in group II are fixed in 4% glutaraldehyde in PM.} \\
\text{(b) Average number of microtubule profiles per unit-cross-sectional area of muscle is charted, including each standard error of the mean, for six dogs and three guinea pigs. Hatched bars represent paired samples from a single dog. Groups I and II are the same as for Fig. 3a.} \]
FIGURE 4 (a) Cross section of cardiac muscle at M band that shows cross-sectional microtubule profile in center of myofilament bundle. $\times$ 280,000. (b) Cross section of cardiac muscle at I band that shows two cross-sectional profiles of microtubules which are closely spaced but separated by typical clear zone of cytoplasm. One profile is ~6 nm from the outer membrane of a mitochondrion. Bar, 0.1 $\mu$m. $\times$ 280,000. (c) Cross section of cardiac muscle at Z band that shows two cross-sectional profiles of microtubules (arrows). The 10-nm filaments are marked by arrowheads. $\times$ 50,000. (d) Cross section of cardiac muscle at Z band that shows a curving, longitudinal profile of a microtubule. Bar, 1 $\mu$m. $\times$ 50,000.

FIGURE 5 (a) Longitudinal section of canine cardiac muscle that shows sigmoid shape of two microtubule profiles between two myofilament bundles. One profile (arrow) can be followed beyond a sarcomere length. Note grazing profiles of SR tubules and cross-sectional profile of a microtubule near I band (arrowhead). Bar, 1 $\mu$m. $\times$ 42,000. (b) Longitudinal section of canine cardiac muscle that shows long straight SR tubule and parallel microtubule profile oriented diagonally across the sarcomere. Note that at the upper right the microtubule curves before passing out of the plane of section and that this microtubule profile may be considered part of a helix with a steep pitch. Note also the three profiles running approximately along the M band with a sigmoid shape. The top sides of the three microtubules, rather than the usual cut surfaces which reveal the two walls of each microtubule, are seen. Bar, 1 $\mu$m. $\times$ 56,000.
Figure 6 Longitudinal section of canine cardiac muscle fixed at room temperature in Millonig's phosphate buffer with paraformaldehyde-glutaraldehyde. Longitudinal profiles of microtubules are seen in adjacent I bands at the level of the SR tubules. One profile is parallel to a budding mitochondrion. Note fenestrated collar of SR at the middle of the top sarcomere (FC) and no visible diagonally oriented microtubule across the middle of this sarcomere, but diagonally oriented microtubules in adjacent areas. Bar, 1 μm. × 30,000.
Longitudinal sections of canine cardiac muscle that show longitudinal profiles of microtubules. (a) and (d) are from hearts fixed in phosphate-buffered paraformaldehyde-glutaraldehyde at room temperature. (b), (c), and (e-g) are fixed in PM. (a) shows an exceptionally straight profile which extends over two half-sarcomeres. (b) shows a microtubule profile with a shallow curve. Microtubule profiles in (c) and (d) suggest an intermediate pitch while those in (e) and (f) suggest a steeper pitch. (g) shows two similar longitudinal-microtubule profiles in adjacent sarcomeres. A similar pitch would be predicted for the long-range ordering of these microtubules. (a-f) Bar, 1 μm × 25,000, (g) Bar, 1 μm × 30,000.
FIGURE 8 An artist's sketch of cardiac muscle that illustrates the various profiles of microtubules seen in the present study. A, A band; I, I band; M, M band; Z, Z band; FC, fenestrated collar; ID, intercalated disc; Mt, microtubule; Mt, mitochondrion; SR, sarcoplasmic reticulum; T, T-tubule.

exactly perpendicular to the plane of section. Few cross-sectional microtubule profiles are seen—both because curving microtubule profiles oblique to the plane of section are difficult to recognize in sections 50-80 nm thick, and because the microtubules curve at different levels with respect to the A bands of the myofibrils.

For a repeating helical network, the same sigmoidal shapes will be seen across each sarcomere of the same myofibril in grazing sections which contain the SR if the network is static and is related to the shape of the myofibril. In the simplest case, the myofibril is round, has a diameter of 1 \( \mu \text{m} \), and the microtubule has a specific unchanging pitch. For a given surface profile of a single sarcomere, then, a given sigmoidal shape will be predicted. In actual fact, we are dealing with myofilament bundles of different shapes from round to ribbon that vary in size.

By picking an arbitrary unit of surface \( 2.3 \times 1 \ \mu \text{m} \), we found at least five different sigmoidal repeat units predicted by the shape of the micro-
Microtubule profiles actually observed in single sarcomeres at rest length. For example, the shallow curve in longitudinal section (Fig. 7a) suggests a helical wrapping over a distance of at least three sarcomeres. The steeper curve in Fig. 5b suggests that the distance traversed may be two sarcomeres. We saw the same sigmoidal shapes in longitudinal sections grazing the myofibril surface in guinea pig cardiac muscle as we did in dog cardiac muscle, even though the myofibril diameters were very often smaller in the guinea pig heart. We were unable to relate the helical pitch of the microtubule arrangement on the myofibril surface to the shape of the myofibril.

By studying thin sections of muscle, we were able to see at most two microtubule profiles which had a similar arrangement in two adjacent sarcomeres. However, diagonally arranged microtubules on the surface of the myofibrils were frequently seen. The cardiac muscle cell is organized into repeating units and the same components are seen in each unit. It is likely, then, that the microtubules exist throughout the myocardial cell in the space between the myofibrils, and that they form a well-organized network. The data suggest at least two groups of microtubules: those arranged near the nucleus, and those near the myofibrils.

**DISCUSSION**

The distribution of microtubules observed in the present study is compatible with several known functions for microtubules. The presence of microtubules along the fiber axis is consistent with the asymmetric cardiac muscle cell and with previous observations of microtubules in muscle. Simpson et al. reported, in a review of cardiac fine structure, that microtubules may run in any direction, but generally stay more or less parallel to the fiber axis (26). Fischman has observed in his study of myofibril formation in embryonic chick skeletal muscle that microtubules are always oriented with the long axis parallel to that of the cell (7). However, Van Winkle and Schwartz have recently shown that microtubules in all three fiber types of adult skeletal muscle are found in at least three orientations (29). Warren has shown in a study of myogenesis in vivo in frog skeletal muscle that the microtubules are required for maintenance of muscle shape and play a role in the orderly arrangement of myofibrils (30). The disruption of the microtubules by colchicine is correlated with the loss of a longitudinal asymmetry in the myoblasts and myotubes of the regenerating muscle in the frog tadpole tail.

The significant numbers of microtubules that we found in mammalian adult heart muscle suggest that the microtubules persist after the formation of myofibrils and that they may continue to perform a cytoskeletal function. Cardiac microtubules have four of the five characteristics listed by Porter (24) for a microtubule system which influences cell shape: They are distributed unevenly according to a pattern, are oriented, possess a tendency toward straightness, and are limited in length. We do not know to what extent they are associated with microtubule-organizing centers and the location of these anchor points.

We do not know the significance of the helical repeat. If a single arrangement of microtubules is assumed, the different pitches observed could reflect a dynamic system in which the microtubules move. The microtubules may serve a mechanical function by providing an opposing force to the constant-volume behavior of the sarcomeres, or an opposing force to the tendency of the myofibrils to split into smaller ones. Helical arrays have been observed in association with the elongation of the nucleus and concentration of chromatin in developing sperm (15, 18). In these cells, a squeezing force has been suggested for the double-helical arrangement (18). However, the microtubules are much more numerous and more closely spaced around the sperm nucleus than they are around individual filament bundles in the muscle cell. Calculation of the possible forces for a helical array would depend on a good estimate of the number of microtubules in heart muscle. Until the optimal conditions for preservation of intact heart microtubules are characterized, it is likely that the number of microtubules observed in the present study is a conservative estimate of the actual number. The fact that we find no significant improvement in the length or number of microtubule profiles in a PM effective in other cell types supports this interpretation.

The several different pitches observed can be interpreted as more than one helical repeat and may mean several different arrangements of microtubules. Different functions can be carried out by the microtubules. One of these functions may be an organizational role for the addition and subtraction of sarcomeres in hypertrophy and atrophy of the adult muscle. The helical arrangements may have significance for the long-range ordering of myofilament bundles and (or) mem-
branes of the SR. This is an attractive hypothesis because Peachey and Eisenberg have recently presented evidence for a spiral arrangement of T-systems and striations of frog skeletal muscle (23).

Microtubules were consistently found in close association with mitochondria and SR as well as with the myofilament bundles. Since a consistent association of microtubules with specific organelles is found when microtubules function in intracellular transport, we examined the microtubules in relation to the membranes of the mitochondria and SR. In longitudinal sections, we sometimes saw finger-like processes of mitochondria parallel to a longitudinal profile of a microtubule, and the distance between the mitochondrial membrane and the microtubule appeared to be fairly consistent. However, measurements of cross sections of canine cardiac muscle did not show any consistent distance between cross-sectional profiles of mitochondria and microtubules. We did not see any clear examples of bridges between the mitochondria and microtubules but did observe a few between SR membranes and microtubules. We saw several examples of a long, straight profile of an SR tubule parallel to a microtubule for a distance of >1 μm.

It is intriguing that a significant number of microtubule profiles are seen in the I band on either side of the Z band near the 10-nm filaments. The thin filaments contain actin and tropomyosin (13), and the Z band contains alpha-actinin (28). These same proteins have been seen in close proximity to microtubules that are found in cultured cells (8) and are involved in transport phenomena (3). Coated vesicles, also believed to play a role in transport (19), are also seen near the T-tubules in this same region (4). Microtubules near the nucleus in cardiac muscle change in orientation with changes in the shape of the nucleus and, as suggested by Ferrans and Roberts (6), the microtubules may play a role in maintenance of the non-round nuclear shape. In addition, the microtubules may provide structural support to the nucleus to keep it in the center of this asymmetric cell.

Our findings of microtubules after both conventional and special fixation procedures suggest that microtubules are more numerous in adult muscle than previously recognized. Their location in the cell suggests that they may perform more than a cytoskeletal function in cardiac cells.

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