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Stereological Measurements of Cardiac Ultrastructures Implicated in Excitation–Contraction Coupling

(sarcotubules and T-system)

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ABSTRACT **Electron micrographs of osmium-fixed left ventricles from 200-g female rats were analyzed by stereological techniques. By the use of equations developed by H. Sitte it was possible to determine volume fractions of organelles and absolute membrane areas per unit cell volume for cellular membrane systems implicated in excitation–contraction coupling. The fractions of cell volume were: mitochondria 0.34, myofibrils 0.481, T-system 0.012, total sarcotubules 0.035, other 0.13. The membrane areas per unit cell volume ($\mu m^2/\mu m^3$) were: external sarcolemma 0.27, external sarcolemma + T-system 0.34, total sarcotubules 1.3. Diads made up 0.08 of sarcotubular volume and 0.12 of sarcotubular membrane area. 0.14 of the external sarcolemmal membrane area was involved in diadic complexes with underlying subsarcolemmal cisterns.**

The ultrastructures thought to be responsible for the physiological phenomena of excitation–contraction coupling and relaxation in heart muscle have been extensively described (1–4). Nevertheless, there is as yet no quantitative information about the fractions of cell volume occupied by these structures, or about the areas of the various membranous surfaces involved in the calcium movements associated with the physiological phenomena (5–7). Although such quantitative structural estimates have been available for some time for frog skeletal muscle (8, 9), the complex geometry of mammalian heart muscle has so far prevented the gathering of comparably reliable data on mammalian heart muscle. We have recently overcome this difficulty by the application of stereological techniques to electron micrographs (10). In this paper we report the results of measurements on rat ventricular myocardium, with particular emphasis on the ultrastructures implicated in excitation–contraction coupling and relaxation.

Ventricles from 200-g female Sprague–Dawley rats were fixed by perfusion with isotonic Krebs–Henseleit solution (pH 7.2) modified to contain 32 mM osmium tetroxide. The fixed left ventricular myocardial wall was prepared for electron microscopic examination as previously described (11) except that embedding was in Araldite instead of Epon. Slightly oblique cross-sections giving a gray interference pattern were stained with permanganate and lead by the method of Reedy (12). This stain allowed the preparation of exceptionally thin sections with high contrast and without serious problems arising from superposition of organelles. The electron microscope was calibrated daily with a Fullam carbon replica cross-grating (21,600 lines per cm). Photographic prints of slightly oblique cross-sections (final magnification 8600×) were subjected to both point counting and line integration by covering them with transparent sheets of thin plastic on which a square grid (1 cm/side) had been photographically imprinted. For measurements of the sarcoplasmic reticulum we used a finer grid (0.32 cm/side, line thickness 0.080 mm) and a final magnification of ×30,000; the grid and print were illuminated by placing them horizontally on an x-ray viewing box. After completion of the initial measurements at the lower magnification, the grid was rotated 19°, the measurement was repeated, and the results obtained at the two orientations were averaged. Areas including a transverse cell boundary or a nucleus were excluded. Additional measurements were made until doubling the area counted did not significantly alter either the mean or the dispersion of the data obtained and until it was clear that the data obtained conformed to a normal distribution.

The fraction of cell volume occupied by a cellular component i was determined by the relation $V_i/V_{cell} = P_i/P_{cell}$, in which P_i is the number of points falling on the component i and P_{cell} is the total number of points falling on the myocardial cells within the 451 cm^2 area of the print. The surface/volume ratio of the cells was calculated from the relation $A_{ESL}/V_{cell} = (\pi/2)C_{ESL}/(a\,P_{cell})$, in which A_{ESL} is the membrane area of the external sarcolemmal envelope, C_{ESL} is one half the total number of intersections of the external sarcolemma with the vertical and horizontal lines of the grid, and a is the length of one side of the small squares on the measuring grid divided by the final magnification of the print. The ratio of external sarcolemmal plus T-tubular membrane area $A_{(ESL+TS)}$ to cell volume was calculated from the relation $A_{(ESL+TS)}/V_{cell} = (\pi/2)C_{(ESL+TS)}/(a\,P_{cell})$, in which $C_{(ESL+TS)}$ is one half of the total number of intersections of the external sarcolemma and T-system with the vertical and horizontal lines of the grid. The fraction of sarcotubular membrane area (A_{ST}) occupied by diads was computed from the relation $A_{diad}/A_{ST} = C_{diad}/C_{ST}$, in which C_{diad} and C_{ST} are, respectively, the number of intersections with the membranes lining the diad and the entire sarcotubular system. For the purposes of calculation, the volume of the T-system was counted as lying within the cells.

Measurements of sarcotubular volumes and membrane areas had to be made at high magnification to obtain adequate resolution; such measurements could therefore sample only a small area. At this high magnification the procedure adopted was to count the points P_{ST} and intersections C_{ST} on the sarcotubules, and also the points falling on all the myofibrils in the print, P_{MF}. From these values the sarcotubular volume

TABLE 1. *Contributions of various structures to cell volume* of rat ventricle†*

Fraction of cell volume					Volume of diads/Total sarcotubular volume
Mitochondria	Myofibrils	T-system	Total sarcotubules	Other	
0.34 ±0.01	0.481 ±0.009	0.012 ±0.002	0.035 ±0.002	0.13	0.08 ±0.02

* Exclusive of nuclear and perinuclear volume and intercalated disk.

† Total points counted were 17,541 for sarcotubules and 10,100 for all other components. In this and the following table data are mean ±SE, the number of prints measured being 10 for sarcotubules and 20 for all other components. Tables 1 and 2 are data from the same heart. Data from two other hearts, though less extensive, were not significantly different.

V_{ST} and membrane area A_{ST} per unit volume of myofibril V_{MF} were calculated according to the equations $V_{ST}/V_{MF} = P_{ST}/P_{MF}$ and $A_{ST}/V_{MF} = (\pi/2)C_{ST}/(a\ P_{MF})$. $V_{MF}/V_{cell} = P_{MF}/P_{cell}$ was determined separately at low magnification, and the quantities V_{ST}/V_{cell} and A_{ST}/V_{cell} were derived by combining these values with the data obtained at the higher magnification. The combination of point counting with lineal integration used in these procedures was that proposed by Sitte (13).

Table 1 presents the contributions of various structures to the volume of ventricular cells. The Poche *et al.* (14) values for mitochondria and myofibrils agree well with those obtained for rat ventricles with a similar technique. Comparison with reported values for frog skeletal muscle of the twitch-type (8) indicates that for rat ventricular cells the fractional volume of the T-system (0.012) is larger and the fractional volume of the sarcotubular system (0.035) is smaller than the corresponding values of 0.003 and 0.13 in skeletal muscle.

Table 2 presents the membrane area per unit volume for various membrane-limited structures. The contribution of T-tubular membrane to the total sarcolemmal surface area is strikingly smaller than that in frog skeletal muscle (Peachey, ref. 8), in which the T-tubular membrane area exceeds that of the external sarcolemma by a factor of seven; this difference is to be expected from the fact that the diameter of rat ventricular cells is about 10% that of the frog muscles described by Peachey. Peachey also found that for frog skeletal muscle cells the total sarcotubular membrane area (in cm^2) was $2.7 \times 10^4\ r$ per cm^2 of external sarcolemma. Assuming a right circular cylinder with $r = 50\ \mu m$, this can be converted to the units of Table 2 to give a figure of 5.4 $\mu m^2/\mu m^3$ cell volume. Peachey indicated that his estimates might be in error by a factor of 2–3; the sarcotubular membrane areas per unit cell volume of rat ventricular muscle is therefore smaller than that for frog twitch muscles, a conclusion in accordance with the qualitative impression. In addition, the contribution of terminal cisterns to the total sarcotubular volume in frog skeletal muscle was 0.38; this value substantially exceeds the corresponding value for rat ventricular muscle.

TABLE 2. *Membrane area per unit cell volume ($\mu m^2/\mu m^3$)* for various membrane-limited structures*

External sarcolemma†	External sarcolemma† T-system	Total sarcotubules	Area of diad membrane/Total area of sarcotubular membrane
0.27 ± 0.01	0.34 ± 0.01	1.3 ± 0.1	0.12 ± 0.02

* Columns 1–3.

† 0.14 ± 0.01 of the area of external sarcolemmal membrane was involved in diadic complexes with underlying subsarcolemmal cisterns.

14% of external sarcolemmal area was involved in diadic complexes with underlying subsarcolemmal cisterns. It has previously been suggested by one of us (15) that the presence of subsarcolemmal cisterns in mammalian ventricular muscle may account for the fact that subthreshold stimulation of discrete areas of the external cell surface fails to elicit a localized contraction of underlying sarcomeres (16). The relatively large fractional area occupied by subsarcolemmal cisterns (Table 2) is consistent with this interpretation.

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