

A MORPHOMETRIC STUDY ON THE NEXUS OF RAT CARDIAC MUSCLE

ALEX MATTER

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ABSTRACT

A morphometric study of the nexus of rat cardiac muscle was carried out. The nexus surface of one intercalated disk of one 15 μm thick fiber is found to range between 47 μm^2 and 94 μm^2 , the latter value taking into account the maximal underestimation caused by tangential sectioning. Dividing the lower, minimal value by the surface of the observed subunits (90 Å periodicity), one obtains for one intercalated disk 6.7×10^5 subunits, each of them assumed to be permeated by a central pore. These pores are thought to be equivalent to intercellular channels in a recently proposed model. Taking our morphometric and recently reported physiological values, this model is examined for its consistency with a low resistance pathway between cardiac muscle cells.

INTRODUCTION

A nexus (8) is a specialized contact zone between the plasma membranes of two homologous and closely apposed cells, being observed in a wide variety of animal tissues. Nexuses are described as gap junctions in conventional electron microscopy, the outer leaflets being separated by a 20 Å cleft (6, 12, 21, 22, 29). It is an interesting model for the study of cell-to-cell interactions, since it is widely accepted that the nexus represents the substrate of electrotonic coupling observed between cells of certain tissues (1, 2, 4, 5, 9, 11, 14, 17, 25, 30, 33, 34). A geometrical model has been proposed (25, 26) which suggests that the nexus is the site of channels where substances of low molecular weight pass between the coupled cells (26, 28, 34). Nexuses display distinct geometrical patterns (honeycombs) after various treatments, such as permanganate fixation (32), negative staining (3), and treatment with lanthanum (12, 29). The periodicities observed correspond to the globular subunits seen in freeze-etched material (7, 12, 16, 22, 27). The working hypothesis of this report is based on two assumptions: (a) the nexus is the morphological

counterpart of electrotonic coupling; (b) the nexus is built up in geometrically well-defined subunits, each subunit contributing equally to the coupling process.

It is, therefore, of interest to know the average nexus surface of one intercalated disk; this would allow one to calculate the number of subunits of one intercalated disk by dividing the nexus surface by the cross-sectional area of one subunit based on the dimensions derived from freeze-etching preparations (7, 12, 16, 22, 27). These values might finally render possible the study of the permeability properties of one subunit. The discussion deals with this latter point, using the data and calculations of S. P. Weidmann and J. A. S. McGuigan.

MATERIALS AND METHODS

Adult white male rats (Wistar) were used. Right cardiac ventricles were either excised immediately after sacrifice and fixed by immersion or fixed *in situ* by perfusion of the whole anesthetized animal: (a) Immersion fixation: either for 2 h at 0°C with 0.6% potassium permanganate (PP) buffered with Veronal

acetate (18), or for 2 h at 0°C with 2% phosphate-buffered osmium tetroxide (23) (OT); (b) Perfusion fixation: with phosphate-buffered 3% glutaraldehyde (GA) followed by immersion fixation for 2 h in 1% phosphate-buffered osmium tetroxide (10).

For each fixation method, five rats were used. A small longitudinal strip was excised from each right ventricle. This strip was then cut into five blocks. This procedure was the same, regardless of fixation, except that in the immersion fixations PP and OT, the still beating ventricles, were cut in a drop of chilled fixative, whereas in perfusion fixation (GA) the blocks were cut after the completion of perfusion. Dehydration with graded alcohol concentrations and embedding in Luft's Epon mixture (19) were performed under the same conditions for all specimens. The twenty-five blocks of each different fixation were cut with glass knives and stained with lead citrate (31). A last group of GA-fixed specimens was double-stained with an aqueous 5% uranyl acetate solution and lead citrate (GA + U). A Philips EM 300 microscope was used throughout this study.

Goniometry

Sections were cut as thin as possible and turned in the goniometer stage for optimal orientation of the nexus parallel to the tilt axis. Series of photographs comprising a nexus that had been turned from -45° to $+45^\circ$ provided the data to determine how much a nexus could be inclined (with respect to the optical axis) and still be recognized as such. This estimation was used to determine approximately the error in counting nexus intersections. This is of some importance, since blurred regions, where the membranes of the intercalated disk are cut tangentially, have been counted as nonnexus regions.

Morphometry

Morphometry was performed using the methods of Weibel et al. (36). All micrographs were taken at the same magnification (8,900) regardless of the orientation of the intercalated disk (calibration of magnification by means of carbon grating replicas, Ernest F. Fullam, Inc., Schenectady, N. Y.). The sample consisted of 300 micrographs of GA+U- and 50 micrographs of PP-, OT-, and GA-fixed specimens. Each micrograph was taken from a different intercalated disk.

The micrographs destined for morphometrical analysis were enlarged three times. A transparent sheet of film with an engraved pattern of 5-mm wide squares was superimposed on the photograph, corner to corner. The intersections of the intercalated disk with the grid were counted with a separate tally of nexus and nonnexus intersections. The percentage obtained in one micrograph was then plotted as one

observation. Mean values were calculated and tests of significance were carried out, to test the hypothesis that the three fixation methods yielded observations of one homogeneous population.

It became apparent, however, that one micrograph could not be considered a representative sample, because too many of them contained no or very few nexus intersections (Fig. 2 a). We therefore reevaluated the data by pooling five consecutive micrographs to form one sampling unit.

RESULTS

The fine structure of nexuses is known to vary according to the technique employed for fixation (6, 21). There are, however, common features: the overall width averages 180–200 Å; the thickening of the inner membrane leaflets with respect to other regions of the cell membrane and the strict parallelism of the two membranes are seen at lower magnification thus rendering the recognition of perpendicularly cut nexus easy and precise. Problems arise in tangential sections, where both nonnexus and nexus regions appear blurred. Since a limit between a sharp and a blurred appearance of membranes cannot be established, it was first decided to count all blurred regions as nonnexus regions. By this procedure, the nexus surface will be underestimated. By goniometrical analysis it can be shown (Fig. 1, inset) that a nexus can be recognized up to an inclination of 40–50°, on an axis perpendicular to the section. The maximum underestimation would therefore be 50%, but probably it is lower since the majority of the photographs showed clearly defined, perpendicularly cut intercalated disks (Figs. 1, 3).

The morphometric measurements yield the following results. If each micrograph is plotted as a single observation, the relative nexus surface expressed as percentage of the total surface of the intercalated disk shows, with all three methods of fixation, a surprisingly wide distribution, ranging from 0 to about 50% relative nexus surface, clearly indicating that one micrograph is not a representative sample. All three distributions differ greatly from normal distributions (Fig. 2 a). The mean values (10.6% for PP, 13.3% for OT, and 8.8% for GA) are not significantly different.

In order to form representative sample units, we have pooled the data obtained on five consecutive micrographs. This eliminated "empty" data points, and the data were symmetrically distributed around the means which showed

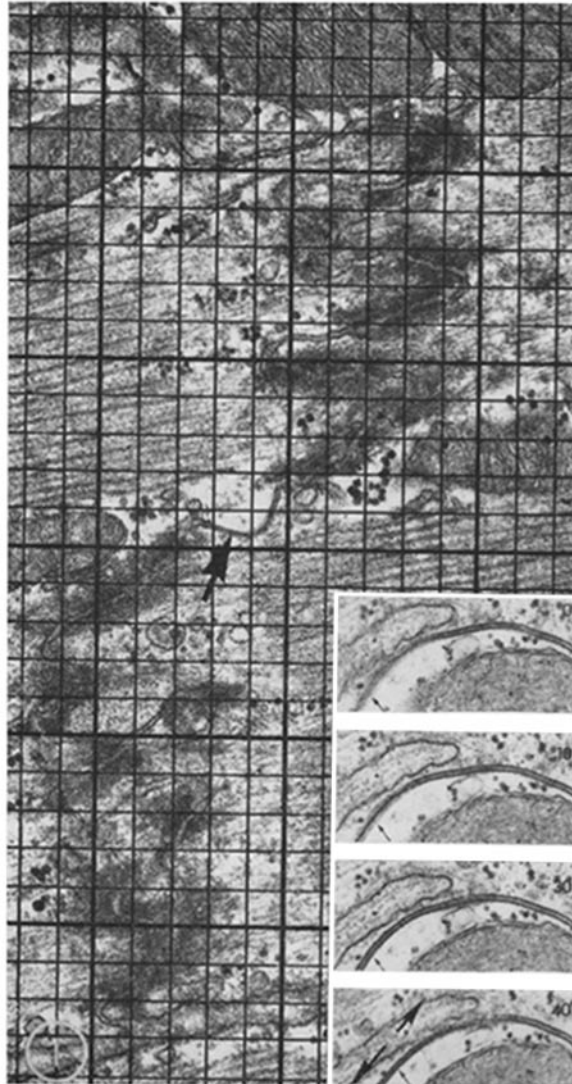


FIGURE 1 Intercalated disk of right cardiac ventricle, GA+U material. This photograph shows the actual magnification of the intercalated disk as well as the size of the grid to obtain the morphometric data. Arrow indicates a nexus. *Inset*: A nexus turned in the goniometer stage from 0° (top) to 40° (bottom). The tilt axis is given by the double arrow on the bottom micrograph. Small arrows point to corresponding spots. $40\text{--}50^\circ$ appear as a limit value by which a nexus can be inclined with respect to the optical axis and still be recognized. This leads to a maximal underestimation of the nexus surface of 50%. Fig. 1, $\times 26,700$. *Inset*, $\times 45,000$.

normal distributions and no significant difference between pairs of the three groups of observations (Fig. 2 b; OT, PP, and GA).

Since the GA material seemed to yield the best preservation, it was chosen for an extended measurement of the nexus surface, in this case double stained (GA+U) to further increase

the contrast of the membranes. The mean value of 300 observations is 7.5% (Fig. 2 b, GA + U) and does not differ significantly from the previously obtained values for PP, OT, and GA. (GA + U vs. GA: $p = 0.42$, GA + U vs. PP: $p = 0.20$, GA + U vs. OT: $p = 0.10$). The absolute value of the nexus surface for one fiber

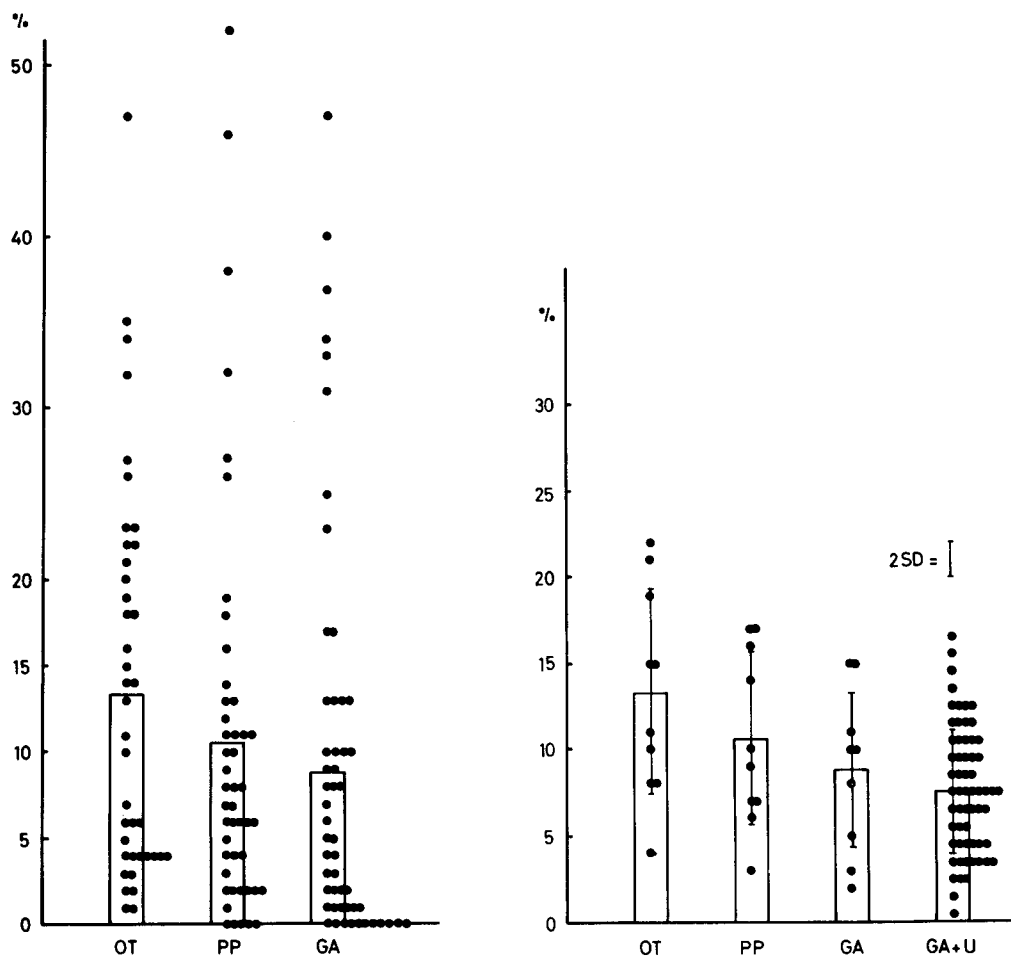


FIGURE 2 Fig. 2 a (left hand side of Fig. 2) shows the three groups of observations (OT, PP, GA), the ordinate showing the percentage of nexus surface with respect to the surface of the intercalated disk (= relative nexus surface). Each point represents one micrograph, the columns represent the mean values. The samples are not representative. Fig. 2 b (right hand side of Fig. 2) is plotted as in Fig. 2 a. Same samples as before, except for GA+U. The micrographs have been pooled at random to groups of five. By this means, representative sampling is obtained. There are no significant differences between the four groups of observations.

of an arbitrary diameter of $15 \mu\text{m}$ (20) can now be calculated. The cross-sectional area of a cylindrical fiber of this diameter is $177 \mu\text{m}^2$. The undulation of the membrane at the intercalated disks brings about a sizeable increase in membrane surface (Fig. 3), which can be estimated by the following reasoning.

On a longitudinal section, the plane cross-sectional area is represented by a straight line trace, whereas the area of the intercalated disk is related to the curved trace of the disk section.

The actual disk surface, S_d , is related to the cross section of the muscle, S_m , as

$$\frac{S_d}{S_m} = \frac{I_d}{I_m} \cdot \frac{4}{\pi}$$

where I_d and I_m are intersections of the test line system (Fig. 1) with the disk trace and the perpendicular straight line, respectively. The factor $4/\pi$ accounts for the spatial curvature of the disk. In ten determinations, the ratio of intersections was found to vary between 2 and 4

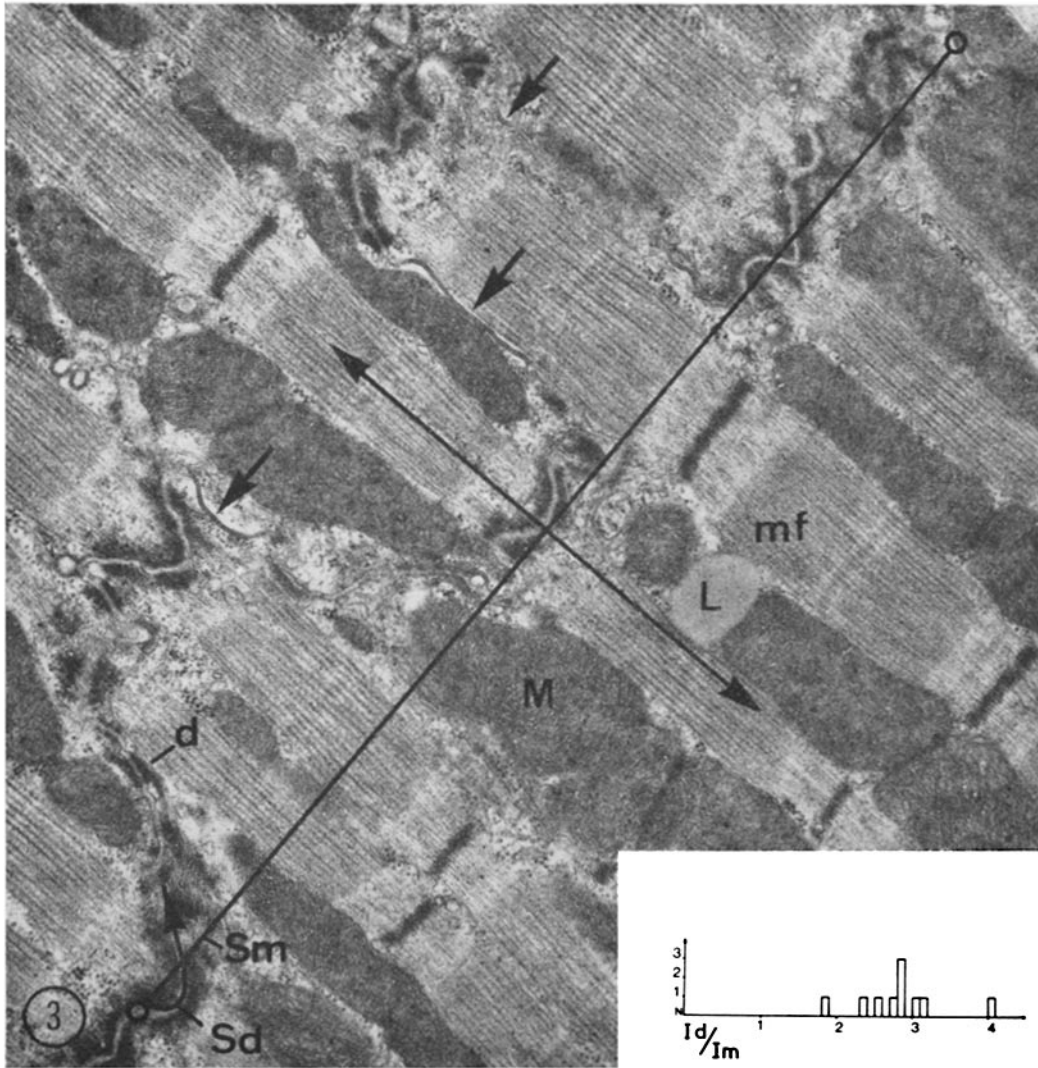


FIGURE 3 GA material. *L*, lipid droplet, *M*, mitochondrion; *mf*, myofibrils; *d*, desmosome. The arrows point to nexuses. *Sd* and *Sm* give the relation between intersections at the line of the intercalated disk and its projection situated perpendicularly to the long axis of the fiber. The resulting factors are plotted in the inset. The mean factor (2.8) allows calculation of the increase in the membrane surface at the intercalated disk due to their undulation. $\times 22,000$.

(Fig. 3), the average being 2.8. This yields a ratio of disk surface to cross section of 3.56.

For a heart muscle fiber of a diameter of $15 \mu\text{m}$ and a cross-sectional area of $177 \mu\text{m}^2$, the surface of the intercalated disk should therefore be about $630 \mu\text{m}^2$. With an average of 7.5% of the disk surface covered by nexus, the surface of nexus connecting two cells of this size must be of the order of $47 \mu\text{m}^2$. This value may be under-

estimated by 50%, at most, due to inclination of part of the disks, as discussed under Materials and Methods.

DISCUSSION

Two major difficulties have been encountered during this study: (a) evaluating partially obliquely sectioned membranes may introduce systematic errors; (b) measuring relatively in-

frequent membrane specializations, which (to be identified properly) required work at high magnification, which allows only a small sample to be analyzed on one micrograph, and therefore does not necessarily yield representative samples.

The first difficulty was partly overcome by determining, with the goniometer stage, correction factors for a possible underestimation of relative nexus surface. The problem of representative sampling could be eliminated by pooling data from individual micrographs.

Random pooling of five micrographs to form one observation changes the frequency distribution of data points from an asymmetrical to symmetrical distribution with reasonably small standard deviations. It was shown that the four methods of preparation (PP, OT, GA, and GA + U) yielded values for relative nexus surface which did not differ significantly although the relative nexus surface appeared to be somewhat lower in GA and GA + U material. The value for GA + U (7.5%) is in substantial agreement with that of Spira (35), who obtained, with a different morphometrical technique, 6% nexus surface in intercalated disks of dog auricles. Spira also noted the wide distribution of the values of nexus surface of intercalated disks and mentioned the possibility of heterogeneity between different ventricular fibers, a possibility which cannot be excluded by the present study.

It is now widely accepted that the nexus is composed of a hexagonal array of globular subunits (3, 7, 12, 22, 27, 29, 32). The model of the nexus that is most consistent with currently available morphological and physiological data proposes a pore in the center of each subunit which could represent an intercellular diffusion channel, isolated from the extracellular space (22, 25, 26).

Our morphometric data, together with recent physiological measurements, allow us to examine the hypothesis as to whether or not this model is consistent with a low resistance pathway connecting heart muscle cells. We have deliberately taken the minimal values least favorable to this argument. The number of globular subunits in one intercalated disk is calculated in the following way: assuming a center-to-center periodicity of 90 Å of the hexagonal array, one obtains a surface of 7,020 Å² for one "hexagon" or subunit. Considering the nexus surface of 47 μm², this yields 6.7 × 10⁵ "hexagons" (and an equal number of channels) for one intercalated disk of

15 μm fiber. Assuming a diameter of the intercellular channel of 10 Å (25) which is probably somewhat lower than the actual value (22, 26, 28), one obtains a cross-sectional channel area of 78.5 Å², yielding a total channel area of about 0.53 μm², which corresponds to about 0.33% of a cross section of one 15 μm fiber.

Intercalated disks offer a resistance to the flow of longitudinal current which adds to the resistance of myoplasm. The question arises as to whether the geometry described by the present quantitative data can provide for a low resistance pathway between adjoining cells. The plausible assumption that K ions are the most important intracellular carriers of charge is supported by a comparison of the longitudinal diffusion coefficient for ⁴²K and the internal longitudinal electrical resistance of bundles of cardiac fibers (37, 38). The following calculation takes into account the value for intracellular potassium in cat papillary muscle (24), 150 mmol per liter of fiber water, assumes free mobility of K⁺ and thus a specific resistance for the movement of K⁺ alone of 85 Ω cm at 37°C (13). Furthermore, the assumption is made (see below) that the channels are filled by aqueous solution.

The resistance of a membrane area of this fluid, 200 Å thick and 1 cm² in cross section is obtained by dividing the specific resistance by the number of such layers per centimeter distance, thus 85 Ω cm/5 × 10⁶ cm⁻¹, or 1.7 × 10⁻⁴ Ω cm². Taking into account that about 0.33% of this layer consists of holes, the rest of insulating material, the specific disk resistance (= resistance of one cm² of folded disk) is obtained as 1.7 × 10⁻⁴ Ω cm² divided by 0.33 × 10⁻², or 0.051 Ω cm². With a cell length of 120 μm and thus 80 disks per centimeter (18) the disks would contribute 4.1 Ω cm to the total resistance of the intracellular phase.

This is a very low value when compared to the figure of 470 Ω cm as obtained by electrical methods for the lumped longitudinal intracellular resistance (disks plus myoplasm; 35). As indicated above, the present calculation of disk resistance is based on some simplifying assumptions. In reality, both the mobility and concentration of K ions within a 10 Å channel will certainly be different from that in free solution, e.g. as a result of negative or positive charge on the walls of the channels. While there is no way to assess these complicating factors the conclusion seems nevertheless justified that the pres-

ent data are in agreement with the idea of a low-resistance pathway between cardiac cells, allowing the propagations of action potentials by local circuit currents.

The present value calculated for specific disk resistance, $0.051 \Omega \text{ cm}^2$, lies well below the range obtained by electrical methods and the method of ^{42}K diffusion with cardiac tissue, $0.25 \Omega \text{ cm}^{-6}$ (15, Table I), which is not surprising in view of the simplifying assumptions underlying the present calculation.

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