Mitochondrial regular arrangement in muscle cells: a “crystal-like” pattern

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Recent studies have shown the existence of multiple specific functional interactions among mitochondria, sarcoplasmic reticulum (SR), and myofibrils in permeabilized muscle fibers (5, 14, 30, 34). Namely, endogenous ATP has been shown to be more efficient than exogenous ATP in maintaining calcium uptake into SR (14). In addition, kinetic studies have shown a direct supply of endogenous ADP from ATPases to mitochondria (30, 34). Such interaction can be explained by the existence of localized intracellular diffusion restrictions (28, 39). A mild treatment of the fibers with trypsin leads to the removal of these diffusion restrictions, and at the same time, distribution of mitochondria in the fiber is changed from regular arrangement in the control to random distribution after the treatment (28). Similarly, in ischemic hearts, various alterations in mitochondrial function such as the significant decrease in maximal respiration rate and half-saturation constant for ADP were observed in parallel with the changes in structural organization of the cardiac muscle cells (7, 16). These experimental results suggest that there is a direct link between regulation of muscle cell energetics and structural organization of the cell (28).

According to ultrastructural electron microscopic studies, mitochondrial position in the muscle cells is rather regular and depends on the muscle type. In the heart muscle, mitochondria are arranged in a longitudinal lattice between the myofibrils and are located within the limits of the sarcomeres (33, 36). The correlation between sarcomere and mitochondrial length is preserved during the heart muscle contraction or when the muscle is stretched (21). In white and red skeletal muscles, mitochondria either are arranged in pairs on both sides of the Z line or form columns in the longitudinal direction in interfilament space (22, 23). Thus the arrangement of mitochondria is rather repetitive and similar for each sarcomere. According to studies in which the fluorescence recovery technique was used, mitochondria are morphologically and functionally heterogeneous in a number of cells (10). In cardiac muscle, mitochondria present single poorly branching organelles in normal conditions (3). In hypoxic conditions, cardiac mitochondria can fuse together to form gigantic mitochondria that are longer than several sarcomeres (37). This is one of the examples showing that mitochondrial morphology is a dynamic process that is regulated by the balance of fission and...
fuscin, and this balance can be changed by such interventions as hypoxia (10, 27).

In all of these studies, however, mitochondrial distribution was not analyzed quantitatively. Such quantitative description is required to relate the structural organization of the muscle cells to interactions between mitochondria and intracellular ATP-consuming systems, and thus to the intracellular energy cross talk. In addition, fixed preparations have been used in the cited ultrastructural studies, and depending on the procedures used in freezing and preparing the fibers, the fixation might influence the results (26).

The aim of this work was to analyze quantitatively the arrangement of mitochondria in heart and skeletal muscles. In this work, confocal images of nonfixed cardiomyocytes and fibers from soleus and white gastrocnemius muscles were analyzed using an algorithm for this purpose. The position of mitochondria relative to each other in different muscles was described using probability density functions. The results show ordered (crystal-like) arrangement of mitochondria with different distribution functions in various muscle cells. This approach may be used for analysis of structural and functional changes in muscle cells in different physiological and pathophysiological states.

METHODS

Animals

Adult Wistar rats (male \( \sim 300 \) g and female \( \sim 240 \) g) were used in all experiments. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Preparation of Skeletal Muscle Fibers

The fibers were prepared from adult female Wistar rat white gastrocnemius and soleus muscles. Fibers were stored and imaged in solution A (see below for composition) in the presence of exogenous substrates (5 mM glutamate and 2 mM malate). The preparation of the fibers was described previously (29).

Preparation of Isolated Cardiomyocytes

Intact ventricular cardiomyocytes were isolated from adult male Wistar rat heart as described by Kay et al. (15).

Confocal Microscopy

Two independent methods were used to visualize the mitochondrial position in the nonpermeabilized muscle fibers and cardiomyocytes. Imaging of mitochondrial calcium using rhod-2 AM. Rhod-2 AM is a cell-permeable fluorescent probe for mitochondrial matrix calcium. Nonpermeabilized muscle fibers were incubated in Multidish 24 wells (Nunc, Roskilde, Denmark) with 5 \( \mu \)M rhod-2 AM in solution A for \( \sim 1 \) h at 4°C in the presence of exogenous substrates (5 mM glutamate and 2 mM malate). Rhod-2 AM has a rhodamine-like fluorophore whose excitation and emission maxima are 557 and 581 nm, respectively, making it convenient to use with argon and krypton lasers as an excitation source.

Imaging of mitochondria using MitoTracker Red CMXRos. MitoTracker Red CMXRos is a derivative of x-rosamine and specifically binds to mitochondria. The fluorescence of this dye was measured (excitation and emission maxima at 579 and 599 nm, respectively). Nonpermeabilized muscle fibers were incubated in Multidish 24 wells (Nunc) with 100 nM MitoTracker Red CMXRos in solution A for 2 h at 4°C in the presence of exogenous substrates (5 mM glutamate and 2 mM malate). Cardiomyocytes were incubated with MitoTracker Red CMXRos for 45 min at 4°C in flexiPERM chambers (Vivascience, Hanau, Germany) in solution A with 5 mM glutamate and 2 mM malate.

Muscle fibers and cardiomyocytes were mounted in flexiPERM slides (Vivascience) in the presence of solution A as follows. Muscle fibers were put on a slide and stretched slightly before the ends of the muscles were fixed by attaching flexiPERM micro12 to the slide. Solution A was then added to each chamber of flexiPERM micro12 that contained the fiber. In the case of cardiomyocytes, flexiPERM micro12 was attached to the slide first, and then solution A and cardiomyocytes were added to a chamber.

All preparations were imaged using a confocal microscope Leica DM IRE2 (Leica Microsystems, Heidelberg, Germany) with a \( \times 63 \) water-immersion objective lens (NA 1.2) at 25°C.

Proteolytic Treatment of Isolated Cardiomyocytes With Trypsin

Isolated cardiomyocytes were permeabilized by the addition of 50 \( \mu \)g/ml saponin under the microscope within the flexiPERM micro12 chamber. After 15 min of permeabilization, 0.2 \( \mu \)M trypsin (Sigma, St. Louis, MO) was added. Proteolytic treatment of trypsin was stopped after 25 min by the addition of 0.025 mg/ml trypsin inhibitor (Sigma). As a result of inhibition, the trypsin reaction was effectively stopped, as judged by the disappearance of movement of mitochondria as observed using a confocal microscope.

Solutions

Solution A contained (in mM) 2.77 \( \text{CaK}_2\text{-EGTA} \), 7.23 \( \text{K}_2\text{-EGTA} \) (free calcium concentration \( \sim 0.1 \) \( \mu \)M), 6.56 \( \text{MgCl}_2 \), 0.5 DTT, 53.3 \( \text{K-MES} \), 20 imidazole, 20 taurine, 5.3 \( \text{Na}_2\text{ATP} \), and 15 phosphocreatine, pH 7.1 adjusted at 25°C. All reagents were purchased from
Mitochondrial pairs (arrows in insets each cardiomyocyte. In soleus and white gastrocnemius (B), mitochondria are rather regularly spaced, forming a mesh covering cardiomyocytes and the skeletal muscle fibers are oriented almost horizontally. Cardiomyocytes and soleus fibers were preloaded with MitoTracker Red Fig. 2. Representative confocal images of nonpermeabilized cardiomyocytes (A), and white gastrocnemius fibers (B), soleus fibers (C). In these images, cardiomyocytes and the skeletal muscle fibers are oriented almost horizontally. Cardiomyocytes and soleus fibers were preloaded with MitoTracker Red CMXRos; white gastrocnemius fibers were preloaded with rhod-2. In cardiomyocytes (A), mitochondria are rather regularly spaced, forming a mesh covering each cardiomyocyte. In soleus and white gastrocnemius (B and C, respectively), mitochondria form rows running across the fiber. These rows are formed by mitochondrial pairs (arrows in insets in B and C). In some images, “column-forming” mitochondria (22) can be identified (arrowheads in insets in B and C). In gastrocnemius, long mitochondria running in pairs in the transverse direction were seen occasionally (asterisks in insets in C). Sizes of images: A, 59.5 × 59.5 μm; B, 94.3 × 94.3 μm; B, insets, 14.3 × 7.1 μm (bottom left) and 7.8 × 7.8 μm (bottom right); C, 112 × 112 μm; C, insets, 6.9 × 6.9 μm (top left), 6 × 4.55 μm (bottom left), and 8.2 × 7.6 μm (bottom right).

Quantitative Analysis of Mitochondrial Positioning

To analyze how mitochondria are positioned relative to each other, we used confocal images of the skeletal muscle fibers and cardiomyocytes with easily distinguishable mitochondria. Each image was rotated until the muscle fiber or cell was oriented in a vertical direction, as judged by eye. Next, the centers of mitochondria were marked manually. For each mitochondrial, the closest mitochondrial centers were found among mitochondria in several sectors as shown in Fig. 1. The relative coordinates of the closest neighbors were then computed.

Before the analysis was performed, the relative coordinates of the closest neighbors were corrected as follows. In cardiomyocytes, the local myofibrillar orientation was estimated and accounted for. Because the contractile apparatus in the cardiac muscle is widely branching, one has to take into account such organization of the muscle before the distances between any two points in the cell are measured (36). For each mitochondrial center, we approximated local myofibrillar orientation by fitting with a straight line the positions of the mitochondrial center and the centers of its two neighbors found in the sectors aligned along the fiber. The fitting was performed using the least-squares method. The obtained line was considered a local orientation of myofibrils, and all of the neighboring mitochondria were rotated to orient the line vertically. The relative coordinates obtained after rotation were stored and analyzed statistically.

In soleus and gastrocnemius, mitochondria form lines that are not exactly perpendicular to fiber orientation (see Fig. 2, B and C). Because the angle between the lines formed by mitochondria and the fiber orientation was different in different fibers and even in the different sections of the same fiber, we transformed the relative mitochondrial positions as follows. First, for each mitochondrial center, we approximated local orientation of the “mitochondrial line” by fitting with the straight line the positions of the mitochondrial center and the centers of its two neighbors found in the sectors aligned along the fiber. The fitting was performed using the least-squares method. Second, new relative coordinates of mitochondrial neighbors were computed by reducing the coordinate value along the fiber by the factor $\cos \alpha$, where $x$ is the relative coordinate of the neighbor mitochondrion in the transverse direction and $\alpha$ is an angle between the transverse direction and the found local “mitochondrial line” direction. Thus the mitochondrial neighbors were sheared to form the “mitochondrial line” that was perpendicular to the fiber. The relative coordinates obtained after such transformation were stored and analyzed statistically.

The statistical analysis was performed by computing the distribution function of the distance between the centers of neighbor mitochondria as well as by computing probability density functions. Both functions (distribution and probability density) were computed for each sector separately. Thus integration of the probability density function over a sector gives a probability equal to one. Probability density function was calculated by dividing each of the sectors with a mesh consisting of $0.1 \times 0.1$-μm boxes and counting the amount of mitochondrial centers within each of the boxes.

In the analysis, the values are expressed as means ± SD. The programs developed for this analysis were written in Python and are available on request.

RESULTS

Representative confocal images of nonfixed intact cardiomyocytes and skeletal muscle fibers are shown in Fig. 2.

CARDIOMYOCYTES

A representative confocal image of a nonpermeabilized cardiomyocyte with marked mitochondria is shown in Fig. 3. In Fig. 4, the distances between mitochondrial centers taken from the image in Fig. 3 are analyzed. According to our analysis (Fig. 4A), the distances between mitochondrial centers are smallest in the direction transverse to the fiber. The largest distances are in the diagonal direction (angle 45°). The distribution can be presented in a radial plot, where the average distance between mitochondrial centers is related to the direction between mitochondria. In this plot, the distances between the centers are given as the distances from the reference point (coordinates 0, 0) plotted in the direction corresponding to each sector (Fig. 4B). From inspection of the radial plot (Fig. 4B), it is clear that mitochondrial centers are not distributed randomly but are arranged according to some regular pattern. Indeed, if
mitochondria were distributed randomly, the mean distance as well as the distribution functions would not depend on direction, and in a radial plot, the centers would have been aligned along a circle.

In the analysis presented in Fig. 4, only the distances between mitochondrial centers within each sector (see Fig. 1 for definition of the sector) are taken into account. To distinguish the distributions of mitochondrial centers in each of the sectors, we computed the probability density functions for mitochondrial centers in each of the sectors (Fig. 5A) using confocal images (n = 10) of cardiomyocytes with a total of 1,820 mitochondrial centers marked. According to the computed probability density function, the mitochondrial centers are packed in certain areas within the sectors. The areas with the highest probabilities are aligned along a rectangle with the longer side of the rectangle oriented along the fiber (Fig. 5A).

Because the distances between mitochondrial centers are not the same in the directions along and perpendicular to the fiber, a considerable number of mitochondrial centers are located near the borders of 45° sectors (Fig. 5A). Taking into account that the distance between mitochondrial centers in the diagonal direction is usually larger than that in the longitudinal or transverse direction, the mitochondrial centers in the diagonal direction are not accounted for by our algorithm if the center is positioned in a different sector. To avoid such interaction of mitochondria in different sectors, the sectors were adjusted as follows: the sectors in the longitudinal direction were taken as equal to 30°, those in the transverse direction as 60°, and those in the diagonal direction as 45°. The probability density functions corresponding to adjusted partitioning of the neighboring mitochondria to the sectors are shown in Fig. 5, B and C.

The regular (crystal-like) arrangement of mitochondrial centers is clear from the mean positions of mitochondrial centers (Fig. 5C). The mean positions are aligned along a rectangle, with longer distances between mitochondrial centers along the fiber. Using the same partitioning of the sectors as in Fig. 5, B and C, the following distances between mitochondrial centers were found: 1.97 ± 0.43 and 1.43 ± 0.43 µm in the longitudinal and transverse directions, respectively. These distances are significantly different (Welch test, P < 0.001), with the difference in the mean distances in the longitudinal and transverse directions equal to 0.52–0.59 µm (95% confidence interval).

**Soleus**

In skeletal muscle, mitochondrial arrangement is different from that in cardiomyocytes, with most of the mitochondria arranged in pairs in soleus (Fig. 2). In our analysis, we marked only mitochondria that were seen as spots in the image (indicated by arrows in Fig. 2B) and ignored the long mitochondria, which were connecting the mitochondrial pairs in the longitudinal direction (marked by arrowheads in Fig. 2B). This approach was used because it is impossible to distinguish whether the connection between pairs of mitochondria is formed by separate but not resolved mitochondria or by long extensions of mitochondria within the pairs. An example confocal image of soleus fiber with marked mitochondrial centers is shown in Fig. 6. Because the arrangement of mitochondria in soleus is different from that in cardiomyocytes, there are large differences in the distribution of mitochondrial centers relative to each other in these muscles. These differences were taken into account as follows.
To determine how the neighboring mitochondria should be partitioned to represent the spatial distribution of mitochondria in soleus, we analyzed in detail the distribution of all marked mitochondria shown in Fig. 6. As a first step of our analysis, we found, for each marked center, the neighboring mitochondria shown in Fig. 6. As a first step of our analysis, in soleus, we analyzed in detail the distribution of all marked mitochondria shown in Fig. 6. As a first step of our analysis, we found, for each marked center, the neighboring mitochondria that were positioned within 5 μm in the longitudinal and transverse directions, i.e., within a 5 × 5-μm box. The size of the box was selected to cover more than one sarcomere length in the muscle. Next, the probability density function describing the distribution of mitochondrial neighbors was computed (Fig. 7A). Because this probability density function describes not only the closest mitochondrial neighbors (as in Fig. 5) but also all neighbors that lie in the 5 × 5-μm box, there are several maxima of probability density function in each direction. Because these maxima are distributed quite symmetrically relative to the reference point (cross in Fig. 7), it is sufficient to recognize the origin of the maxima located in one-fourth of the plot only. The origin of marked maxima in the positive transverse and longitudinal directions is indicated in the scheme in Fig. 7B. For example, maximum 5 in Fig. 7A corresponds to the neighboring mitochondria in the transverse direction. Because both mitochondria within the pair usually have neighbors in the transverse direction, this maximum is considerably larger than maximum 6 in Fig. 7. Other maxima produced by both mitochondria within the pair are maxima 3 and 8. Note that these maxima are considerably larger (presented by brighter spots in Fig. 7A) than the others (compare with maxima 2, 4, 6, 7, and 9) in the map (Fig. 7A). In the longitudinal direction, the maxima (maxima 1–4) are clearly separated from each other because of the small relative variation in the distances between mitochondrial centers in this direction. In the transverse direction, the maxima corresponding to the closest neighbors (maxima 5–9) are distinguishable, but not as clearly as in the longitudinal direction. The maxima corresponding to the neighboring mitochondria that are further away in the transverse direction from the center are fused together, leading to the formation of lines in Fig. 7A. This indicates that the relative variation of distances in the transverse direction is considerably larger than in the longitudinal direction.

To describe the distribution of mitochondria in soleus, we determined the distribution of mitochondrial neighbors corresponding to maxima 1, 2, 5, 6, and 7 in Fig. 7 and their symmetric reflections. Indeed, mitochondria positioned near these maxima determine the characteristic distance in the transverse direction (distance 5 in Fig. 7B) as well as both characteristic distances in the longitudinal direction (distances 1 and 2 in Fig. 7B). The probability density function corresponding to such partitioning of neighbors into sectors is shown in Fig. 8. The probability density function was found by using n = 10 confocal images with a total of 1,820 mitochondrial centers marked. In A and B, different partitioning of the sectors was used: in A, the space was partitioned into sectors of the same size, whereas in B, the partitioning was performed with sectors that were adjusted for analysis of cardiac muscle cell. Note that after such adjustment, the regions with a high density of mitochondrial centers are adrift from the borders of the sectors. In C, the mean positions of neighboring mitochondrial centers are indicated by solid dots for each of the sectors. The area containing 50% of the centers in the sector closest to each mean position is surrounded by a solid line. A similar area corresponding to 75% of the centers is enclosed by a dashed line.
in white gastrocnemius. The distances between the pairs of mitochondria were as follows: 3.33 ± 0.96 and 1.09 ± 0.41 μm in the longitudinal and transverse directions, respectively. According to the Welch test, the mean distance obtained for the transverse direction is considerably larger \((P < 0.001)\) than the same distance in soleus \((0.75 ± 0.22 \mu m, distance 5 \text{ in Fig. 7B})\). The difference between the mean transversal distances in soleus and white gastrocnemius was 0.31–0.38 μm \((95\% \text{ confidence interval})\).

In some confocal images, we were able to distinguish mitochondria within the pairs. According to our data \((10\) images with 85 distances found), the distance between the mitochondrial centers in pairs was 0.61 ± 0.07 μm. Note that the mean value of this distance was considerably smaller \((\text{Welch test, } P < 0.001)\) than that found for soleus \((0.91 ± 0.11 \mu m, distance 1 \text{ in Fig. 7B})\). The difference between the mean distances between mitochondria in pairs in white gastrocnemius and soleus was 0.28–0.31 μm \((95\% \text{ confidence interval})\).

### Alternation of Mitochondrial Organization by Proteolytic Treatment

Mitochondrial arrangement in the cell can be changed dramatically by rather selective proteolytic treatment of permeabilized cardiac muscle cells \((28, 30)\). After the addition of trypsin into solution with permeabilized cardiomyocytes, several drastic changes occur in the structure of the cells. After a period of time required for proteolytic challenge of the cell, cardiomyocytes contract very abruptly, but then the volume of the cell starts to increase slowly, leading to complete digestion of the cell if trypsin is not inhibited \(\text{(results not shown)}\). A representative confocal image after 25-min exposure of the permeabilized cardiomyocyte to 0.2 μM trypsin is shown in Fig. 11.

From a visual comparison of the mitochondrial distribution in the control \(\text{(Fig. 3)}\) and after trypsin addition \(\text{(Fig. 11)}\), the difference is evident: instead of the highly ordered pattern in the control, cardiomyocytes are seen as disorganized clusters after trypsin treatment. Intriguingly, these mitochondrial clusters are still firmly fixed to some intracellular structures. To quantify changes in the distribution of mitochondria within the cell, we computed probability density functions corresponding to the situation after trypsin treatment. When computing probability density function based on the images of cardiomyocytes subjected to prolonged treatment with trypsin, we were not
able to relate the distances between mitochondrial centers to local fiber orientation, because this orientation was not visible (Fig. 11). For reference, the cell border was used instead. As is clear from the computed probability distribution functions (Fig. 12), the regular arrangement of mitochondrial centers relative to each other seems to be the distance between the centers, leading to a probability density function that is almost radially symmetrical (Fig. 12).

**DISCUSSION**

In this work, the arrangement of mitochondria within the muscle was assessed by analyzing quantitatively the distances between mitochondrial centers. To our knowledge, this is the first time the positions of neighboring mitochondria have been characterized by using probability density functions in muscle cells. The analysis of confocal images of nonfixed cardiomyocytes and skeletal muscle fibers by using this method reveals that intermyofibrillar mitochondria are arranged in a highly ordered pattern (crystal-like) with relatively small deviation in the distances between neighboring mitochondria in cardiac and skeletal muscles. Moreover, this arrangement is muscle specific.

According to ultrastructural studies of cardiac muscle, mitochondria occupy 30–35% of myocardial cell volume (6, 19, 32, 36) and are distributed everywhere in the cell (36). Intermyo fibrillar mitochondria usually do not violate the limits of sarcomeres and are located between the zone demarcated by two Z lines (20, 33, 35, 36). Thus, in the longitudinal direction, the distance between the centers of two neighboring mitochondria is expected to be similar to the sarcomere length. From our analysis, this distance is 2 μm and is close to the measured sarcomere length value in the relaxed state (36). In the transverse direction, it has been suggested that all contractile material is within 0.5 μm of mitochondria in cardiac muscle (36). By taking the maximal diameter of a myofibril as equal to 1 μm, we determined that the distance between the centers of neighboring mitochondria in the transverse direction should be larger than 1 μm by the diameter of a mitochondrion. The mean distance found in our study (1.5 μm) suggests that the diameter of mitochondria is at least 0.5 μm in nonfixed preparations.

In skeletal muscle, intermyofibrillar mitochondria are arranged in register with the sarcomere. In red fibers, mitochondria form either pairs on both sides of the Z line or columns on the A-band level (22, 23). In white fibers, most mitochondria are organized in pairs next to the Z line, and rather few are located between Z lines in the form of thin columns (22, 23). The same pattern of mitochondrial arrangement is evident from confocal images of skeletal muscles obtained using specific fluorescent markers (Figs. 2 and 6). In some images, thin links between the bright spots can be identified (arrowheads in Fig. 2, B and C, insets), corresponding to the “column-forming” mitochondria of Ogata and Yamasaki (22). In addition, long pairs of mitochondria running along the I band (see mitochondria marked by asterisks in Fig. 2C) were observed in white gastrocnemius, in agreement with earlier studies (22, 23). In our analysis, we did not consider the thin columns or pairs of...
mitochondria (marked by arrowheads and asterisks in Fig. 2) but marked only the centers of mitochondria within the pairs along the I band. This method was used because it is hard to distinguish whether these long structures are formed by separate mitochondria or long extensions of mitochondria (12) within the marked bright spots in I band-limited mitochondria. Thus the distances analyzed in skeletal muscle represent the distances between the mitochondria forming pairs on both sides of the Z line.

On the basis of our images, we conclude that the pattern of mitochondrial arrangement is similar in soleus (mainly oxidative muscle) and white gastrocnemius (mainly glycolytic muscle). This is in contrast to the differences in the regulation of respiration by exogenous ADP: in oxidative skeletal muscles, the affinity for ADP is very low (apparent $K_m$ is very high), whereas in glycolytic muscle, the mitochondrial affinities for ADP are very high in vivo and in vitro (17, 38). Thus, regardless of the differences in the participation of mitochondria in energy cross talk in these two muscle types, mitochondria are arranged in a similar and highly ordered manner. In the longitudinal direction, the distances between two pairs of mitochondria are similar for soleus (sum of the distances corresponding to maxima 1 and 2 in Fig. 7, $\sim 3.7 \, \mu m$) and white gastrocnemius (distance between the pairs, $\sim 3.3 \, \mu m$) and, according to the ultrastructural studies, correspond to the distances between two Z lines. Because we stretched the skeletal muscle fibers when mounting them on a microscopic slide (see METHODS), the sarcomere length is somewhat larger than attributed to physiological conditions (9) and corresponds to the descending limb in the force-length relationship (24). The main difference between the soleus and white gastrocnemius is in the distances between mitochondrial centers within the same I band. Namely, in the longitudinal direction, mitochondrial centers are considerably closer to each other in white gastrocnemius muscle than in soleus, leading to the reduced width of “mitochondrial band” covering each Z line in white gastrocnemius. In the transverse direction, the distances between mitochondrial centers are considerably smaller in soleus than in white gastrocnemius, pointing to the higher density of mitochondria in soleus.

Most of the ultrastructural studies of mitochondrial arrangement in muscle cells have been performed on fixed and frozen material. Depending on the procedures used in freezing and preparing the fibers, the results of cellular volume assessments can vary $\geq 50\%$ (26). In our study, we were able to analyze the mitochondrial distribution in nonfixed fibers surrounded with solution containing substrates. The fibers or cells were isolated to investigate them using confocal microscopy, but the intracellular environment was kept, similar to the recent studies on nuclear patterning in living muscle cells in the intact animal (8). Because there is no fixation involved, our technique allows us to study quantitatively alteration in mitochondrial distribution in response to changes in the physiological state of the same fiber or cell, i.e., with fully functional mitochondria.

There is an important difference between mitochondrial distributions in cardiac muscle cells observed using electron and confocal microscopy. Usually, the images obtained using electron microscopy from the thin sections of cardiac muscle show larger amounts of irregularities in the position of intermyofibrillar mitochondria than the images obtained using con-

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**Fig. 10.** Distribution of mitochondrial pairs in white gastrocnemius fiber. Images ($n = 8$) were analyzed with a total of 621 mitochondrial pairs marked. In $A$, the probability density function is shown. In $B$, the mean positions of neighboring pairs are indicated by solid dots for each of the sectors. The area containing 50% of the centers in the sector closest to each mean position is surrounded by a solid line. A similar area corresponding to 75% of the centers is enclosed by a dashed line.

**Fig. 11.** Representative confocal image of a permeabilized cardiomyocyte after proteolytic treatment with trypsin. Cardiomyocytes were preloaded with MitoTracker Red CMXRos. Note that after this treatment with trypsin, mitochondria formed clusters. Centers of mitochondria ($n = 83$) within 2 such clusters were marked with small black boxes, as shown. Image size is $35.3 \times 35.3 \, \mu m$. 

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focal microscopy. For example, it is common that there are several mitochondria packed together between sarcomeres (12, 13, 36). The confocal images of cardiac muscle cells show remarkable regularity in arrangement of mitochondria between myofibrils (11). The regular arrangement of mitochondria in confocal images of cardiac muscle has been demonstrated by cross-correlating the fluorescent images of flavoprotein (autofluorescence) and tetramethylrhodamine ethyl ester (mitochondrial inner membrane potential-sensitive dye) (25). Namely, such a cross-correlation map has several peaks that are regularly spaced in the longitudinal and transverse directions. If a central peak in the cross-correlation map represents the coincidence of both images, then peripheral peaks observed in the map indicate the periodicity of the packing of mitochondria along myofibrils (25). Recently, Aon et al. (2) used the regularity of mitochondrial positioning in the cardiac cell by dividing the image of the cell with the grid filled with small squares (\( \sim 2 \times 2 \, \mu m \)) and analyzing the fluorescence within each of the squares. With the use of such an approach, usually one to two mitochondria were observed in each square of the grid (2). The reasons for such a difference between mitochondrial distribution assessed using electron microscopy and confocal microscopy are not clear. One of the possible explanations is that when thin sections are analyzed, as in electron microscopy, a mitochondrion either can be left out of the section by a very small margin or can have several membrane invasions that split a mitochondrion into several mitochondria in the image plane. In addition, fixation procedures used in electron microscopy may play a role here. Alternatively, several mitochondria could be represented by a single bright spot in confocal images because of the resolution limits of confocal microscopy. Whether any of these explanations is correct is not clear and requires further investigation.

Organization of mitochondria in the cardiac muscle cells can be influenced by several challenges. As an example, we followed proteolytic treatment of trypsin, which leads to disarrangement of mitochondria in permeabilized cardiac muscle fibers (28, 30). By using the probability density function describing the distribution of mitochondria, it is possible to analyze quantitatively the transition between a highly ordered pattern in the control and a rather random pattern after prolonged treatment with trypsin. According to our recordings, the transition between a crystal-like pattern (control, Fig. 5) and a radially symmetric one (prolonged treatment with trypsin, Fig. 12) is rather abrupt and goes first through contraction of the cell, followed by relaxation (not shown). These structural changes are most probably related to successive destruction of cytoskeletal components responsible for precise arrangement of mitochondria in the cells. The precise nature of these components is still unknown. Other cardiac challenges, such as ischemia and hypoxia, represent areas of high interest in which the method described in this work can be applied.

Our study has an important limitation. Namely, we used only one section in our analysis and not the series of sections covering a three-dimensional bulk of tissue. In confocal images, the fluorescent signal is detected from a rather thick section, >0.3 \( \mu m \) (given for emission at 488 nm), because of the limitation in resolution. Thus the distances that we computed are projections from three-dimensional space to our image plane, leading to some underestimation of these distances. To overcome this limitation, the series of images must be acquired covering the three-dimensional area of interest and analyzed to reveal three-dimensional organization of mitochondria in the cells. Such an extension of our method is under development.

The regular arrangement of mitochondria in the heart muscle cells is in accordance with the recently described functional interactions between mitochondria and myofibrillar and SR ATPases (5, 14, 30, 34). In the 1960s, the even distribution of mitochondria in the cross sections of heart muscles led to the following suggestion: “Each mitochondrion serves only a very limited area of the myofilament mass immediately surrounding it” (12). As pointed out by Sommer and Jennings (36), the close association of mitochondria with other intracellular structures must not be taken as prime evidence for specific functional interaction, because the amount of mitochondria is very large in the cardiac cells. However, there is kinetic evidence of such interactions from the experiments on permeabilized cardiac fibers: 1) it is impossible to inhibit completely mitochondrial respiration when stimulated by endogenous ATPases using an externally added ADP-trapping system as long as the intracellular structures are kept intact (30, 34); 2) calcium uptake can be enhanced by endogenous rephosphorylation of ADP to ATP by using either endogenous creatine kinase or oxidative phosphorylation compared with exogenous ATP (14). The interaction between mitochondria and ATP-consuming systems can be explained by local intracellular diffusion restrictions that divide the cell into unitary structures containing a mitochondrion and enzymatic systems consuming ATP next to the mitochondrion, such as SR and actomyosin.
ATPases (28, 30, 39). The crystal-like arrangement seen in our study is consistent with this hypothesis and suggests the unitary structure of the studied muscle cells. Implications of such a unitary structure in the regulation of oxidative phosphorylation within units as well as synchronization of events in these units within the cell are not clear and are the subject of active research (1, 2, 31, 40).

Although in the cardiac cells the very precise, crystal-like arrangement of mitochondria is clearly important for regulation of their function and is related to the rigid and precise organization of cytoskeletal structures and sarcromeric proteins, in many other cells the situation is very different and much more dynamic. Randomly organized and changing mitochondrial networks have been described for many types of cells, but always the mitochondrial position is dependent on their connection with the cytoskeleton, notably with the microtubular network and intermediate filaments (4, 27). In addition, cytoskeletal changes that disrupt the mitochondrial distribution can cause cell death (41, 42). The mitochondrial movement is particularly interesting in the neurons, where axonal mitochondrial transport directed by molecular motors can occur along both microtubular or actin filaments to the sites of high ATP demand (18, 41, 42). It may be assumed that, in these cells, the formation of the intracellular energetic units is of a dynamic nature and occurs in a spatially and temporally regulated manner dependent on local energy demand.

In conclusion, our results show that mitochondria, which are situated between the myofibrils, are arranged in a highly ordered crystal-like pattern in a muscle-specific manner with relatively small deviation in the distances between neighboring mitochondria. This is consistent with the concept of the unitary nature of the spatial organization of the muscle energy metabolism. The developed method may be used for relating structural and functional changes in muscle cells in several physiological and pathophysiological states.

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