The Location of Muscle Calcium with Respect to the Myofibrils

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ABSTRACT Autoradiographs have been prepared from frog toe muscles soaked in Ca⁴⁺ and fixed with an osmium-oxalate solution. A majority of the grains over the A bands were over the myofibrils. The grain density over the I bands was greatest over the space between the myofibrils. The significance of this distribution is discussed in the light of previous information about the longitudinal distribution of Ca⁴⁺ in skeletal muscle.

An attractive hypothesis for the coupling of excitation with contraction in skeletal muscle is that depolarization of the membrane causes the release of calcium from some intracellular store. This calcium then initiates the interaction of the contractile filaments. Several different types of evidence support such a mechanism. Muscle models such as isolated myofibrils, glycerol-extracted fibers, and muscle fibers from which the membrane has been removed contract when the calcium concentration is increased to greater than 10⁻⁷ M (1-3). The endoplasmic reticulum of muscle cells contains a system which accumulates calcium and relaxes contracted muscle models simultaneously (4, 5, 11). This system can lower the calcium concentration of a solution to less than 10⁻⁷ M. The depolarization of the surface membrane is conducted to the cell interior along tubular invaginations which lie within a few hundred Angstrom units of the endoplasmic reticulum (6-9).

In an autoradiographic study of the location of calcium in frog skeletal muscle it was shown that most of the exchangeable calcium is localized to the center 0.2 to 0.3 μ of the I band and the part of the A band where the thick and thin contractile filaments overlap (10). The relative concentration of calcium in the two regions could be related to the amount of tension that the muscle was exerting at the time of histological fixation. The greater the tension the greater was the localization to the A band and the smaller the localization to the I band. In these studies it was not possible to distinguish
clearly the borders of the myofibrils. It was, therefore, not possible to comment on the transverse distribution of calcium in these muscles. The observations made, however, were consistent with the calcium hypothesis for excitation-contraction coupling. In these studies the tissue had been sectioned with the knife edge parallel to the longitudinal axis of the fibers. This technique produces a transverse compression of the tissue section but does not decrease the widths of the A and I bands. (This was pointed out several years ago by H. E. Huxley.) It facilitates an analysis of the longitudinal distribution of the silver grains produced by the decay of Ca^{45} in the tissue but impairs any attempt to study the transverse distribution. To obviate this problem, further autoradiographic studies of Ca^{45} in frog skeletal muscle have been made in which longitudinal tissue sections were cut with the knife edge perpendicular to the longitudinal axis of the muscle. In these sections a transverse resolution is favored at the expense of longitudinal resolution.

**METHODS**

The procedure was essentially the same as that used in the previously reported autoradiographic study (10).

Frog toe muscles were isolated and suspended in a constant perfusion bath. After 1 hour of exposure to Ringer's solution the muscles were treated in one of two ways. One group was exposed to Ca^{45} Ringer's for 8 hours, to non-radioactive Ringer's solution for 10 minutes, and then fixed with solutions containing 2 mmoles per liter oxalate and 0.01 to 1.0 per cent osmium tetroxide (10). A K+ contracture was produced in the second group with a modified Ringer's solution containing 50 mM KC1. After 15 minutes, when the muscle had completely relaxed, it was soaked in a Ca^{45} solution of the same composition for 8 hours. At the conclusion of the radioactive soak the muscle was washed with an identical non-radioactive solution for 10 minutes and then fixed with an osmium-oxalate solution. The polarized muscles developed about 40 per cent of tetanus tension and the depolarized muscles about 10 per cent of tetanus tension during fixation. The muscles were dehydrated in ethanol and embedded in butyl methacrylate. Tissue sections about 0.1 μ thick were cut with the knife edge perpendicular to the longitudinal axis of the fibers and were covered with a one grain thick layer of Ilford L4 nuclear emulsion. Tissue and emulsion were stored for 2 to 8 weeks at 4°C and then developed. The autoradiographs were examined with phase contrast optics (100 X N.A. 1.32) and photographs of areas selected for the clear definition of the myofibrils were analyzed for grain distribution. As the silver grains and the tissue section are not in the same plane, it was difficult to photograph the autoradiographs with both grains and tissue completely in focus. Pictures were taken for analysis with the tissue in best focus, and the identification of the grains was confirmed by comparing the photograph with the actual autoradiograph viewed through the microscope.

**RESULTS**

In each autoradiograph, there were areas totalling about 10 per cent of the tissue in which the interfibrillar space could be clearly distinguished from the
FIGURE 1. Autoradiographs of (a) a muscle fixed in osmium without prior depolarization and (b) a muscle fixed in osmium with prior depolarization. The A bands are dark and the I bands are light. The small black dots are silver grains produced by the radioactive disintegration of Ca$^{45}$. The dark line on each figure equals 10 μ. In (a) the inter-fibrillar space in several places has been indicated by pairs of black lines. These photographs were made at an intermediate focus between the one best for the grains and the one best for the tissue. As a result the definition of each is less than can be achieved.
myofibrils. The distribution of these areas bore no relation to position within the whole muscle bundle or within the individual fibers. The thickness of the myofibrils varied from 0.3 to 1.0 μ and the width of the interfibrillar space varied between 0.1 and 0.4 μ. This occurred because the fibrils could not be cut uniformly throughout a section due to their arrangement within a fiber. The ratio of the total myofibril to interfibrillar area on the photographs which were analyzed was 2.6 to 1. Over 1000 grains were counted in 19 autoradiographs (Fig. 1) prepared from 6 muscles of which 3 had contracted in response to K⁺ and 3 had not been exposed to a high K⁺ solution. The location of the center of each grain was determined with respect to the cross-striations and the longitudinal myofibrillar pattern. The myofibril width was divided into four equal parts, and the grains over the center pair were counted separately from those over the pair along the edges. The interfibrillar space was too narrow to be subdivided.

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As in the previous studies, the muscles which developed considerable tension at the time of fixation produced autoradiographs with the highest grain density in the A band outside the H zone. Autoradiographs of muscles which developed little tension during fixation had the highest grain density in the center of the I band. The results of the analysis of grain distribution over areas where the edges of the myofibrils were clear are shown in Table I. This includes the results from both groups of muscles. The grain density in the A band was greatest over the center of myofibrils and the lowest over the interfibrillar space. The opposite was true for the I band.

The A and I bands were divided into five and three equal regions respectively by lines parallel to the bands. Although in all three regions of the I band the grain density was higher over the interfibrillar area than over the adjacent myofibrils, the difference was greater in the central region than in the two lateral regions. The ratio of interfibrillar grain density to myofibrillar grain density in the central region was approximately 2.6, and in the lateral regions averaged together it was almost 1.9. No such difference was seen over the A band.
DISCUSSION

By avoiding the transverse compression of the tissue section that results from orienting the microtome knife edge parallel to the longitudinal axis of the fiber it has been possible to make autoradiographs of skeletal muscle in which the borders of the myofibrils are frequently visible with phase contrast optics. Areas in which the definition was clear were not sufficiently prevalent to permit a separate analysis of each of the two kinds of experiments, but when data from all the experiments were included, a definite transverse pattern of distribution of the grains was apparent.

From the distribution of grains within the A band, it appears likely that all or almost all the A band Ca\(^{46}\) is located within the myofibril. According to the theoretical analysis of the spread of grains around a line source of Ca\(^{46}\), the grain density ought to fall to 70 per cent by 0.1 \(\mu\), to 25 per cent by 0.3 \(\mu\), and essentially reach 0 per cent by 0.5 to 0.6 \(\mu\) (10). If one assumes an average myofibril width of 0.7 \(\mu\) and an average interfibrillar space of 0.2 to 0.3 \(\mu\), then according to the theoretical model the Ca\(^{46}\) concentration is significantly greater in the center of the myofibril than along its edge. A reasonable explanation for this observation is that at least part of the loss of Ca\(^{46}\) which occurred during fixation (5 to 25 per cent) was from the superficial portion of the myofibril (10).

If the same average values for the widths of the myofibrils and the space between them are applied to an analysis of the I band grains, one finds that the degree of localization of Ca\(^{46}\) between the myofibrils is greater than the grain densities within the I band. The reason for this is that the A band Ca\(^{46}\) is localized near the A-I junction and therefore radioactive decay should cause the production of some silver grains in the I band over the myofibrils near the A-I junction. In favor of this conclusion is the fact that a larger percentage of grains over the center of the I band was in the interfibrillar space than of the grains over the edges of the I band. As most of the I band Ca\(^{46}\) is in the center of the band, only a small percentage of the grains over the A band would have been produced by I band Ca\(^{46}\).

The transverse distribution of the grains indicates, then, that most of the A band Ca\(^{46}\) is in the myofibrils and most of the I band Ca\(^{46}\) is in between the myofibrils. The muscle fiber structures which most strongly bind calcium are the myofilaments and the microsomes (12). The latter are thought to be elements of the endoplasmic reticulum (5). One interpretation of these autoradiographic data, therefore, would have the I band exchangeable calcium bound or intimately associated with the endoplasmic reticulum between the fibrils and the A band exchangeable calcium bound to the contractile fila-
ments. Coupled with previous autoradiographic data on calcium in muscle, this suggests that in the relaxed state exchangeable calcium is localized primarily to the lateral cisternae of the endoplasmic reticulum and in the contracted state to the A band filaments. Such an interpretation is consistent with the observations of Hasselbach (13) and Costantin et al. (14) that muscle models soaked in oxalate solutions have large electron-dense deposits in the lateral cisternae. It also supports the calcium hypothesis for the link between excitation and contraction in skeletal muscle.

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REFERENCES