Intracellular Calcium Movements of Frog Skeletal Muscle during Recovery from Tetanus

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ABSTRACT Radioautographs of ^{45}Ca-labeled frog skeletal muscles have been prepared using freeze-dry and vapor fixation techniques to avoid displacement of the isotope during the preparation of the radioautographs. ^{45}Ca has been localized in resting muscles exposed to ^{45}Ca Ringer's for 5 min or 5 hr and in isotopically labeled muscles recovering from tetanic stimulation at room temperature or at 4°C. In muscles soaked at rest for 5 min ^{45}Ca was present almost exclusively in the terminal cisternae. In all other muscles there were three sites at which the isotope was concentrated: (*a*) the terminal cisternae, (*b*) the intermediate cisternae and the longitudinal tubules, and (*c*) the A band portion of the myofibrils. The terminal cisternae were labeled more rapidly than the myofibrils, but both exchanges were accelerated by electrical stimulation. The amount of ^{45}Ca in the longitudinal tubules and the intermediate cisternae decreased with time after a tetanus as the amount in the terminal cisternae increased. It is proposed that electrical stimulation releases calcium from the terminal cisternae and that relaxation occurs from the binding of the released calcium by the longitudinal tubules and the intermediate cisternae. Complete recovery from mechanical activity involves the transport of this bound calcium into the reticulum and its subsequent binding by the terminal cisternae. Resting exchange of calcium occurs primarily between the terminal cisternae and the transverse tubules.

A large body of information indicates that a release of intracellular calcium is critical for the coupling of excitation with contraction of skeletal muscle. The contractile proteins require the addition of about 0.1–0.2 μmole of calcium per g of muscle for the conversion of the muscle from the resting state to the maximally contracted state (11). A shift of this amount of calcium from the region of the terminal cisternae of the sarcoplasmic reticulum to the immediate vicinity of the thick filaments has been demonstrated by radioautographic techniques which used chemical stimulation of the muscle and chemical fixa-

tion (3, 4). Chemical fixation has the limitations of neither being able to stop the mechanical events at any given stage nor to prevent the translocation of calcium in the tissue during the preparation of the radioautograph unless oxalate is included to precipitate the calcium. To avoid these limitations freeze-drying and vapor phase fixation have been used instead of chemical fixation. Radioautographs of isotopically labeled tissues prepared in this way have been used for the study of intracellular calcium movements associated with recovery from mechanical activity. They suggest that the site of calcium storage within the resting cell and the location of calcium reaccumulation from the sarcoplasm following depolarization are separate. Relaxation may occur as a result of calcium binding by the outer surface of the sarcoplasmic reticular membrane rather than by transport across it (13).

METHODS

Frog (*Rana pipiens*) extensor longus digiti IV and semitendinosus muscles which had been pared down to bundles of about 12–15 fibers were used in all experiments. A small mass of tissue was necessary for freezing to be sufficiently rapid to avoid disruption of tissue structure by the formation of sizable crystals. Each end of the muscle was mounted with stainless steel wire on a slender stainless steel rod. The muscle was at least 4 mm from the rod to prevent the cooling of the rod from slowing the cooling of the muscle. The muscle was allowed to recover from the dissection for 60 min in Ringer's solution which contained per liter 1 mmole $CaCl_2$ and 117 mmole NaCl and was buffered at pH 7.0 with 2.15 mmole K_2HPO_4 and 0.85 mmole KH_2PO_4. The muscle was then transferred to another Ringer's solution that had been labeled with ^{45}Ca (specific activity approximately 0.7 mc/ml). The total calcium concentration of the radioactive Ringer solution was approximately 2.0 mM to allow the ^{45}Ca Ringer to be as radioactive as possible. The higher the radioactivity, the shorter the exposure period of the radioautographs need be.

The muscles were exposed to ^{45}Ca for different periods of time. Some were cooled to 4°C during the exposure and the rest were kept at room temperature (25°C). Some muscles were stimulated before being frozen. Each experiment was repeated on at least two additional muscles.

The muscles were frozen in the following manner. Approximately 20–30 sec before the muscle was frozen the tube containing its bathing medium was removed. Sometimes the muscle was dipped into nonradioactive solution for 1 sec to remove the radioactive solution adhering to its surface. A piece of filter paper was placed on the lower tendon of the vertically suspended muscle for about 2 sec to absorb as much of the fluid adhering to the surface of the muscle as possible. This step was important in order to minimize the mass that was to be cooled. Then the muscle was very rapidly immersed in liquid Freon 12, which had been cooled to its freezing point of −160°C with liquid nitrogen. The muscle was frozen before any contraction could occur (20). The frozen muscle was quickly transferred in a tube containing Freon at −160°C to a stainless steel beaker which had been cooled to −85°C in a low temperature chamber. The beaker was loaded with four muscles, and then it was sealed tightly and connected to a

Precision vacuum pump. The beaker temperature was raised to −60°C, and the pump was run with its ballast open until the Freon surrounding the muscles had vaporized. The ballast was then closed, and the muscles were dried for 6 days under a pressure of about 0.5 μm of mercury.

Each vacuum-dried muscle was treated in one of two ways. In the first procedure the pump was turned off, the temperature was raised to −40°C, and the steel beaker with the muscles was opened to a tube containing solid osmium tetroxide which had been previously dried with calcium chloride. The osmium tetroxide sublimated and reacted with the muscles. After 24 hr the osmium was pumped out, and a mixture of 15% methyl methacrylate and 85% butyl methacrylate was added to the steel beaker to cover the muscles. The vacuum was then broken. The histologically fixed muscles were cut up into small pieces about 2 mm long and imbedded in the methacrylate mixture.

The second procedure that was used to imbed the dehydrated muscle made use of the fact that ethanol does not remove the ^{45}Ca from frog skeletal muscle which has been labeled with the isotope (3). The dried muscles were covered with chilled absolute ethanol containing 1 mmole of oxalic acid and 5.0 g osmium tetroxide per liter, and then the vacuum was broken. After approximately 30 min in this solution the muscles were passed through absolute ethanol, cut into 2 mm pieces, and imbedded in methacrylate as described above. No ^{45}Ca was found with a windowless gas flow counter (background 10 cpm) in either the ethanol or the methacrylate used in this procedure. The two imbedding procedures produced essentially the same results.

Gold and gold to purple sections were cut from the methacrylate blocks with a Porter-Blum MT-1 microtome. A solution of 20% acetone in distilled water was used to float the sections off the glass knives. Radioactivity was found in the acetone solution after a large number of sections had been cut. The following experiment was done to determine the mechanism of contamination of the acetone solution. Several ribbons of sections were cut from a block containing a tissue which had been uniformly labeled with ^{45}Ca. Each ribbon consisted of sections of the same thickness, but the range of thickness among the ribbons varied between 0.1 and 2 μ. The acetone solution was emptied after each ribbon had been cut, and its radioactivity was measured with a windowless gas flow counter. The amount of radioactivity was independent of the thickness of the sections and of the length of time during which the sections floated on the acetone solution, but was directly related to the number of sections in the ribbon. The acetone solution contained an amount of radioactivity equivalent to the ^{45}Ca present in one 100 A thickness of the block for each section in the ribbon. It seemed reasonable, therefore, to attribute the radioactivity in the acetone solution to a loss of ^{45}Ca from the block produced each time the block was cut. To minimize the ^{45}Ca carried from the acetone solution with the section when the latter was picked up on a cover slip, the solution was changed frequently. All cover slips were rinsed with fresh distilled water to remove any isotope that had been carried from the acetone solution.

The tissue sections were covered with very thin films of Ilford Nuclear Emulsion L4 (Ilford Ltd., England). The emulsion was exposed to the sections at 4°C and developed in a way that has already been described (3, 4).

For the mechanical studies the muscles were suspended isometrically and their

tension was recorded continuously with a Statham strain gauge (Statham Instruments, Inc., Los Angeles, Calif.) and a Brush recorder (Brush Instruments, Chicago, Ill.). Electrical stimulation was produced through two massive platinum electrodes.

RESULTS

A. *Validity of Technique for Evaluating the Position of In Vivo* ^{45}Ca The interpretation of the experimental data is dependent upon how well the freeze-dry method used in these studies preserved the state of the muscle and prevented any translocation of calcium during the preparation of the radioautographs. Two general criteria have been used to evaluate the degree of success of the method: (*a*) the amount of loss of ^{45}Ca into the various solutions used for preparing the radioautographs; and (*b*) the extent to which extracellular calcium, which is almost certainly the most mobile form of calcium in the tissue, remained in the extracellular space. The absence of radioactivity in either the ethanol solution or the liquid methacrylate indicated that no calcium was lost from the tissue during the preparation of the tissue block. ^{45}Ca did, however, appear in the boat solution behind the glass knife during the sectioning of the tissue (see Methods). The concentration of the isotope depended on the number of sections and not the total amount of tissue. The ^{45}Ca in the acetone solution most probably came from the block material which was removed with the sections but which was not part of them. Such a loss of isotope from the block would not alter the calcium distribution within the tissue. The potential problem of increased background from contamination of the sections with radioactivity from the boat solution was obviated by frequent changes of the boat solution and by brief washing of the sections.

If no translocation of the isotope had occurred during the preparation of radioautographs from muscles which had been exposed to ^{45}Ca Ringer's for a very short time (0.5–5 min), most of the grains should have been over the extracellular space. Fig. 1, a radioautograph of such a muscle, illustrates a much greater grain density over the extracellular space than over the cells. The grains over the cells are due primarily to the oblique pathways of some of the electrons produced by the radioactive decay of ^{45}Ca within the extracellular space. The rate of the decline in grain density from the cell surface inward is consistent with this explanation (3). The higher grain density over the broader parts of the extracellular space suggests that the entire extracellular space of the muscle bundle had not equilibrated with the bathing solution in 30 sec. A higher power radioautograph of the edge of a cell in a muscle exposed to ^{45}Ca Ringer's for 5 min has the largest grain density along the outer surface of the cell (Fig. 2). The density falls by more than 50% over a fraction of a micron at the surface of the cell. This type of grain distribution indicates that the ^{45}Ca was in the extracellular space along the surface of the cell. It furnishes experimental support for the predicted ability to determine the position

of ^{45}Ca with radioautographs made with thin sections and thin emulsion films (3).

Initially, radioautographs of thin sections (600–800A) did not always show the very sharp localization of grains in the extracellular space under conditions

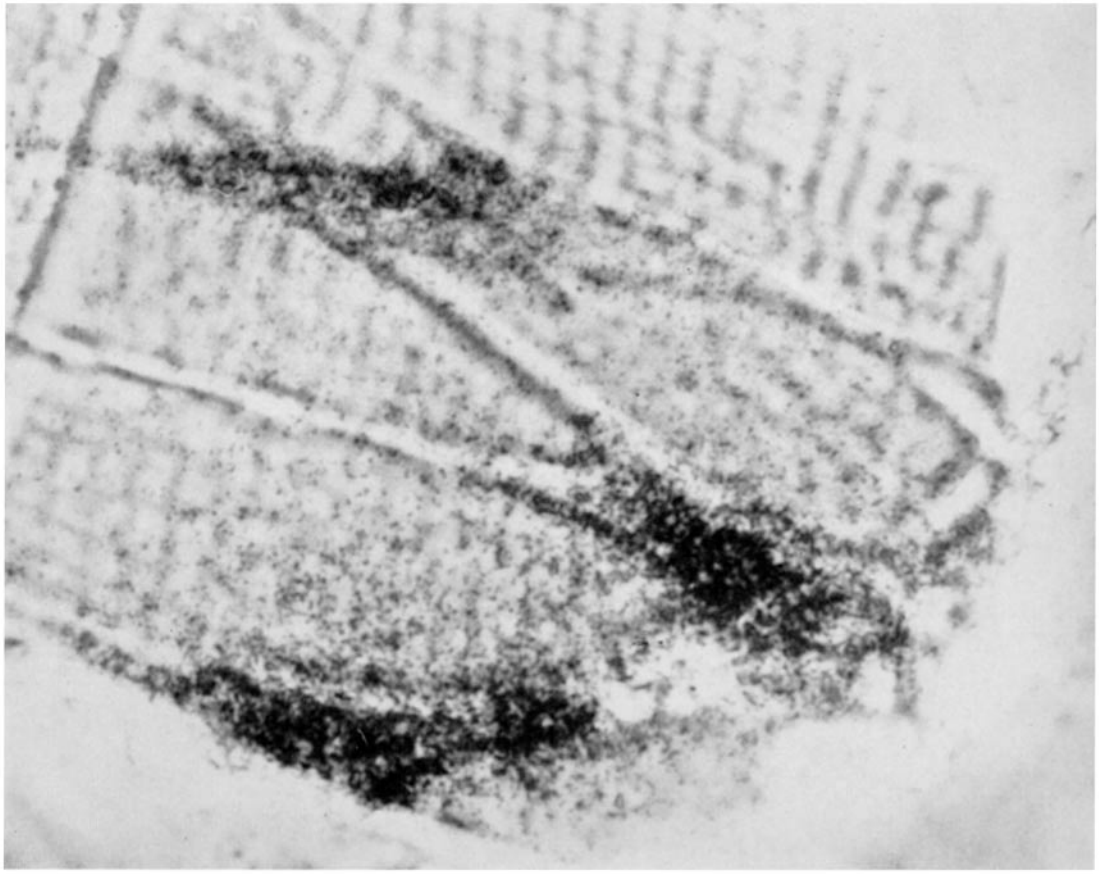

FIGURE 1. Low power radioautograph of a toe muscle soaked in ^{45}Ca Ringer's for 30 sec before being frozen. The grain density is very heavy over the broader portions of the extracellular space. The grain density over the adjacent areas of the cells is much less and is due to the spread of grains around a ^{45}Ca source within the extracellular space. Section 0.2 μ thick.

in which almost all the ^{45}Ca should have been in the extracellular space. Although the grain density over the extracellular space was several times that over the cells, the ratio of the two was lower than expected. When a radioautograph was made from a superficial section of a muscle that had been dipped in nonradioactive Ringer's for a few seconds before it was frozen, the grain density over the extracellular space was very much lower. The short

exposure to nonradioactive Ringer's had considerably lowered the concentration of ^{45}Ca in the extracellular space near the surface of the muscle bundle. The distribution of grains over the cells with either high or low extracellular ^{45}Ca concentrations was very similar except that the background sometimes

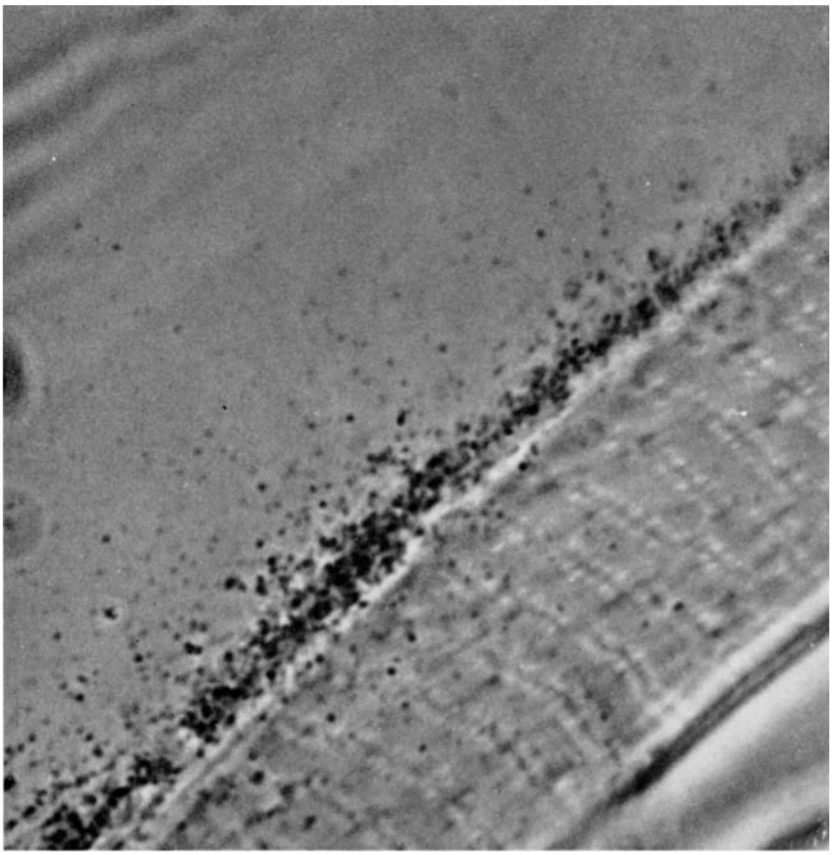

FIGURE 2. A higher power radioautograph of a toe muscle soaked in ^{45}Ca Ringer's for 5 min. The heavy grain density along the surface of the cell is due to ^{45}Ca in the extracellular space. There is a very abrupt decrease in grain density at the cell surface giving a measure of the resolving power for localizing ^{45}Ca. Section 0.1 μ thick.

appeared to be higher where the extracellular ^{45}Ca was higher. These findings suggested that a certain amount of calcium within the tissue section had moved in a random way. This problem disappeared when harder tissue blocks were made with 85% butyl–15% methyl methacrylate instead of 100% butyl methacrylate. It appears that the knife edge distorted the surface of the section as it cut the softer block.

The freeze-dry technique as employed in these studies does, therefore, permit the localization of soluble calcium deposits with reasonable accuracy.

B. *Calcium Distribution in Resting Muscle* The longitudinal distribution of ^{45}Ca in a muscle soaked in ^{45}Ca Ringer's solution for 5 min. is shown in Figure 3 A. The muscle was suspended at approximately 125% of in vivo length to facilitate resolution by increasing the length of the sarcomeres. Since almost all the grains that could be localized transversely were within 0.2 μ of the interfibrillar space, essentially all the intracellular ^{45}Ca was located between the myofibrils. The grain density was highest in the center of the I band and declined sharply with distance from the Z line. Very few grains were present in the A band except in the portion nearest the A-I junction. The rate of decline in grain density from the central region of the I band indicated that almost all this ^{45}Ca was present in the central 0.3–0.4 μ of the I band. From the ratio of the extracellular grain density to the average intracellular grain density and the concentration of calcium in the extracellular space, an uptake of 0.08 μmole per g of muscle over the 5 min of exposure was calculated. The peripheral 1 μ of the fiber was not included in the calculation because the emulsion above it would have been influenced by ^{45}Ca outside the cell.

The longitudinal grain distribution in radioautographs of a muscle soaked at rest in ^{45}Ca Ringer's for 5 hr is shown in Fig. 4, and the transverse distribution is given in Fig. 5. There were two peaks of grain density in the longitudinal distribution. The larger, narrower one was in the center of the I band and was similar to the one seen in the radioautographs from muscles soaked for 5 min. The second peak, which was in the region of the A-I junction, was not present in the short soak radioautographs. The transverse distribution indicates that almost all the I band grains were over the interfibrillar space, and a large fraction of the A band grains were located over the myofibrils.

The results with the 5 hr exposure differed from those with a 5 min exposure in three ways: (*a*) The total ^{45}Ca uptake was greater in the 5 hr soaks; it was equivalent to approximately 0.8 μmole of calcium per g of muscle. (*b*) A significant amount of calcium, about 0.2 μmole per g of muscle, was labeled with isotope in the A band portion of the myofibril. (*c*) There was a second peak in grain density between the myofibrils in the region of the A-I junction.

C. *The Distribution of ^{45}Ca in Muscles Recovering from a Tetanus* Two series of muscles were tetanized for 0.06 min at 50/sec. At this frequency of stimulation the muscle maintained the tetanus with no decline in tension. The grain distribution in a muscle soaked in ^{45}Ca for 5 min at rest, in a muscle fixed 20 sec after the end of a tetanus, and in a muscle fixed 3.5 min after a tetanus is shown in Fig. 3 A–C. The transverse distribution in the muscle fixed 3.5 min after a tetanus is given in Fig. 6. In muscles fixed 20 sec after a tetanus, the transverse distribution was very similar to the distribution 3.5 min after a tetanus in that most of the grains lay over the interfibrillar space. Those grains over the myofibrils were almost entirely in the A band. Several points are worth noting about the differences among these three sets of data. Asso-

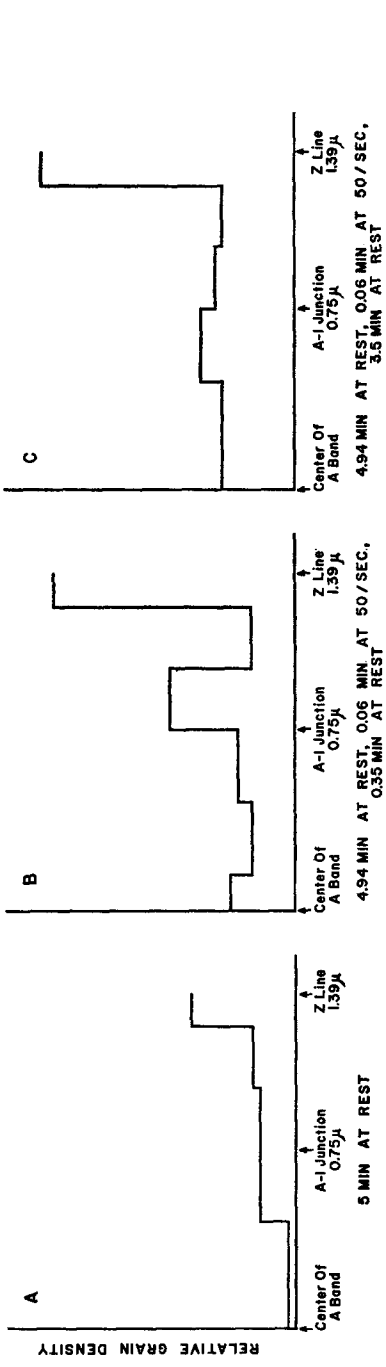

FIGURE 3. The relative grain density (average number of grains per unit area in given regions of the sarcomere) as a function of sarcomere structure is indicated for three different types of experiments. For each experiment the sarcomere was divided arbitrarily into 10 zones and the number of grains counted in each zone. Each graph indicates the distribution of about 1000 grains. Since the extracellular concentration of calcium was the same in all three groups of experiments, a correction for the difference in specific activity of the isotopic soak solutions was made by comparing the absolute grain densities over the extracellular spaces. The ordinate, therefore, represents relative concentration of ^{45}Ca in the various zones of the sarcomere, and the scale is the same for all three graphs. The area under curve 3 A is less than that under 3 B or 3 C because a large uptake of ^{45}Ca occurred during the period of mechanical activity. A, toe muscle has been soaked in ^{45}Ca Ringer's at rest for 5 min and frozen; B, toe muscle soaked at rest for 4.94 min, stimulated at 50/sec for 0.06 min, and soaked at rest for an additional 0.35 min, all in ^{45}Ca Ringer's solution; C, toe muscle soaked 4.94 min at rest, stimulated for 0.06 min at 50/sec, and then soaked for 3.5 min at rest all in ^{45}Ca Ringer's solution. Temperature 25°C.

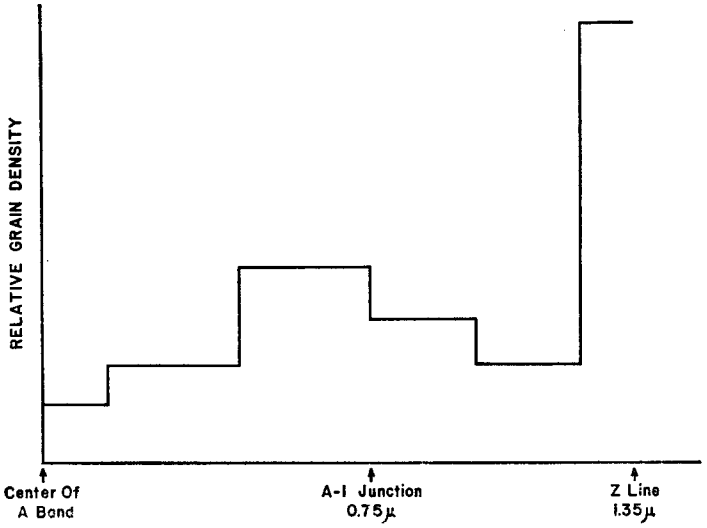

FIGURE 4. The longitudinal distribution of grains in radioautographs of a muscle soaked in ^{45}Ca Ringer's for 5 hr at rest and then frozen. Approximately 1000 grains were counted. Temperature 25°C.

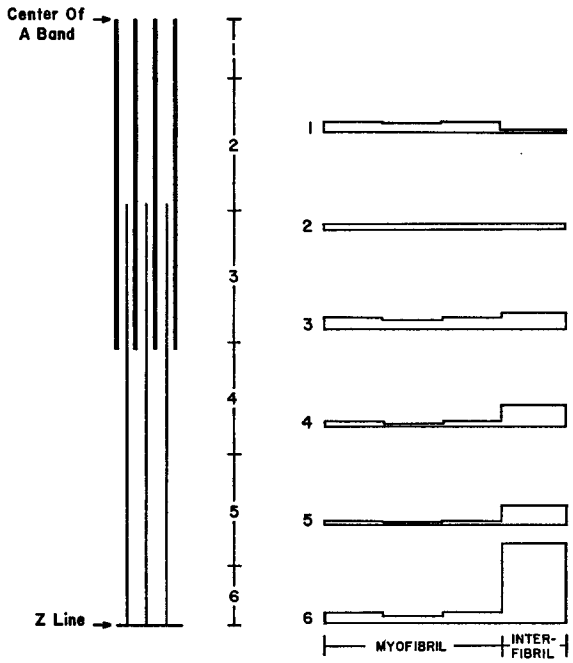

FIGURE 5. The transverse distribution of grains in the same experiment as that of Fig. 4. The sarcomere had been arbitrarily divided into 10 longitudinal and 4 transverse zones for localizing the grains. The block graphs indicate the grain densities in the 24 zones of a half-sarcomere relative to that found over the center region of the I band between the myofibrils. Temperature 25°C.

ciated with stimulation there was an increase in the uptake of ^{45}Ca of 7.2×10^{-10} moles per g of muscle per stimulus, as indicated by the larger area under curve 3B than under 3A. After the period of stimulation there was a significant amount of ^{45}Ca in the A band portion of the myofibril. A peak in the distribution of ^{45}Ca at the A-I junction between the myofibrils was very prominent 20 sec after the end of the tetanus and it had decreased considerably without completely disappearing by 3.5 min after the tetanus.

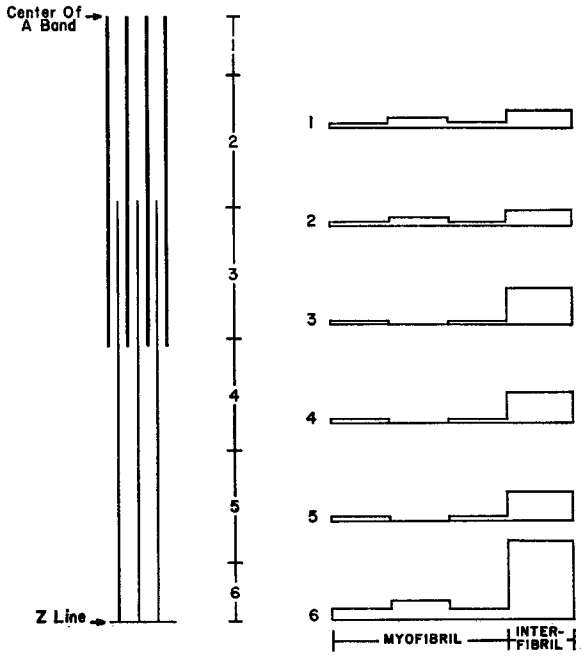

FIGURE 6. The transverse distribution of grains in the same experiment as that of Fig. 3 C (see legend for Fig. 5). Temperature 25°C. 3.5 min post tetanus; total 8.5 min in ^{45}Ca.

A similar set of results was found when analogous studies were conducted at 4°C (Fig. 7–9). Muscles which had been soaked in ^{45}Ca Ringer's at 25°C for 4½ hr and at 4°C for an additional ½ hr were frozen either 30 sec or 10 min after a 0.06 min tetanus. The peak in grain density near the A-I junction was larger and broader 30 sec after the tetanus at low temperature than it was 20 sec after a tetanus at room temperature. The peak at the A-I junction in the low temperature studies became about as small in 10 min as it did in 3.5 min at 25°C.

D. *Contractility in the Posttetanic Period* The contractile properties of muscles recovering from a tetanus were examined in the light of the change in calcium distribution that was occurring at the same time. Toe muscles were

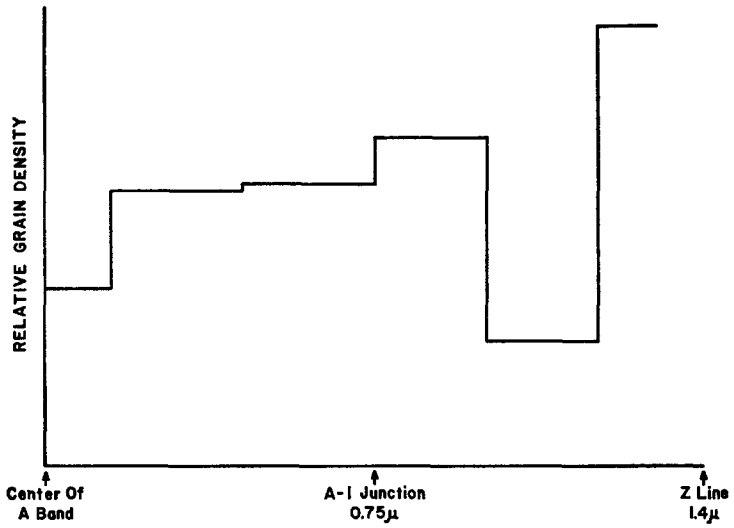

FIGURE 7. The longitudinal distribution of grains in radioautographs of a muscle soaked at rest for 5 hr, stimulated at 50/sec for 0.06 min, and soaked at rest again for 0.5 min, all in ^{45}Ca Ringer's. Temperature 4°C. Approximately 1000 grains counted.

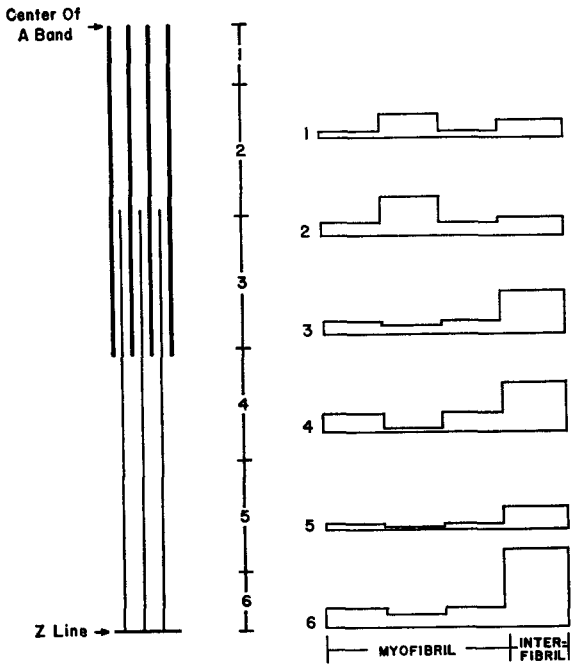

FIGURE 8. The transverse distribution of grains in the same experiment as that of Fig. 7.

stimulated with massive platinum electrodes in the presence of 3 mg % tubocurarine, which eliminated any indirect activation of the fibers through nerve endings. After a 0.06 min tetanus at 25°C, a small potentiation generally appeared on the first posttetanic twitch and reached its maximum level in 10–12 sec. This type of posttetanic potentiation has already been described (21, 22). Occasionally the first one to three contractions were slightly weaker than the pretetanic ones and the potentiation appeared with the fourth twitch. The potentiation elicited under these conditions lasted 3–3.5 min.

FIGURE 9. The longitudinal grain density in a muscle frozen after 5 hr at rest, 0.06 min at 50/sec, and 10 min at rest in ^{45}Ca Ringer's solution. Temperature 4°C. Approximately 1000 grains counted. The longitudinal distribution of grains over just the interfibrillar space was very similar to that in the muscle soaked at rest for 5 hr, and over just the myofibril, very similar to that in the muscle soaked at rest for 5 hr and frozen 0.5 min after a tetanus.

E. *The Localization of Calcium within the Myofibril* In radioautographs of muscles soaked in ^{45}Ca at rest for a very short time, essentially all the grains lay over the interfibrillar space or a narrow region on either side of this space. In radioautographs of muscles that had been either tetanized or had been exposed to ^{45}Ca for long periods of time, however, a substantial number of grains lay over the center of the myofibril within the A band. The localization of these grains over the myofibrils in radioautographs from all muscles which had been either stimulated or soaked for 5 hr at rest in isotopic Ringer's is given in Table I. Grains along the outer edge of the myofibril could have been produced by ^{45}Ca either between or within the myofibrils. The grain distribution over the I band illustrates the former alternative. Grains over the center

of the myofibril, however, should have been produced almost entirely by ^{45}Ca within the myofibril. The grain density over the middle 0.9 μ of the A band was relatively uniform, but the grain density over the part of the A band next to the A-I junction, where the thick and thin filaments overlap, was about one-quarter less. The significance of this difference is enhanced by the very low grain density over the center of the I band next to the A band. There is no indication of grains in the I band produced by ^{45}Ca in the border of the A band. Further study is necessary to determine whether the correlation between grain density and overlap is fortuitous and to learn the significance of the apparently nonuniform exchange of calcium in the region of the thick filaments.

TABLE I

THE RELATIVE DISTRIBUTION OF GRAINS PER UNIT AREA OVER THE MYOFIBRILS*

	A band			I band		
	Center fifth	Intermediate fifth	End fifth	End fifth	Intermediate fifth	Center fifth
Peripheral one-third of myofibril	1.0	1.3	1.5	1.7	1.2	1.5
Central one-third of myofibril	2.0	2.1	1.5	0.1	0.1	0.6

* In experiments in which the muscles were either soaked at rest for 5 hr or stimulated in ^{45}Ca Ringer's solution.

DISCUSSION

A quick freezing technique has made it possible to study the location of even the more mobile forms of calcium in skeletal muscle. In the resting muscle, calcium was localized primarily between the myofibrils in the immediate vicinity of the sarcoplasmic reticulum and to a lesser extent near the thick filaments. In these two parts of the cell the rates of calcium exchange with extracellular calcium differed. Calcium in the region of the reticulum exchanged much more rapidly than the calcium near the thick filaments did, although contraction accelerated the latter more than the former exchange (Table II).

The calcium localized between the fibrils was probably directly associated with the sarcoplasmic reticulum, but its distribution did not simply conform to the volume of the reticulum. Using the figures given by Peachey (6) for the relative surface and the relative volume of the various parts of the sarcoplasmic reticulum of a frog sartorius, one can roughly calculate the relative amount of exchangeable calcium in each part of the reticulum with respect to the volume and the surface area of that part of the reticulum (Table III). The different calcium concentrations (grains per unit volume) in the

TABLE II

CALCIUM COMPONENTS IN FROG TOE MUSCLE

Component	Uptake			Capacity*
	5 min rest	300 min rest	4.94 min rest plus 0.06 min tetanus	
		μmole/g		μmole/g
Reticulum	0.08	0.6	0.18	0.6
Myosin	0.00	0.2	0.02	0.3

Studies performed at 25°C.

* The half-times of exchange used for the calculation of the capacity of the reticulum and myosin were 50 min and 300 min respectively (19).

various parts of the reticulum of muscles soaked for 5 hr at rest show that all the calcium within the volume of the reticulum is not simply in free solution. Calcium binding is probably occurring. The figures expressed in terms of surface indicate that a single type of binding site may be relatively uniformly distributed over the entire reticulum membrane. In view of the nonuniform differences between the resting distribution of calcium in the various parts of the reticulum and the distribution shortly after a tetanus, however (Table III), there are likely to be at least two different kinds of binding sites in the sarcoplasmic reticulum.

These data suggest that the reticulum may bind as much as 0.6 μmole of calcium per g of muscle (Table II). Isolated sarcoplasmic reticulum can bind this much calcium if it is in equilibrium with a free calcium concentration that is higher than can exist in a relaxed muscle (7). A relaxed muscle, however, could tolerate the high intracellular calcium concentration if the calcium were separated from contractile filaments as it might be if the high calcium concentration existed inside the reticulum (1). The precipitation of calcium oxalate crystals inside the terminal cisternae of glycerol-extracted or

TABLE III

DISTRIBUTION OF GRAINS IN THE SARCOPLASMIC RETICULUM*

	Terminal cisternae	Intermediate cisternae	Longitudinal tubules	Fenestrated collar
5 hr rest in ^{45}Ca				
G/unit volume	1.0	1.1	2.5	0.3
G/unit area	1.0	0.6	0.6	0.4
30 sec after tetanus at 4°C in ^{45}Ca				
G/unit volume	1.0	1.8	3.4	0.4
G/unit area	1.0	1.1	1.6	0.3

* With respect to the volume and the surface area of the various parts of the reticulum. The latter values are those found by Peachey (6) in the frog sartorius. The values for the terminal cisternae have been arbitrarily designated as 1.0 and the others expressed relative to them.

skinned fibers by the addition of oxalate (8–10) supports the notion that most of the reticulum calcium in the resting muscle is in equilibrium with the intra-reticulum phase.

It is unlikely that all or even a major part of the calcium in the center of the I band is in the transverse tubule, although it is not possible to be certain of this. The transverse tubules include about 0.3% of the cell volume (6). If the concentration of calcium within the transverse tubule is the same as it is in the extracellular space, the total calcium content of the tubules of muscles soaked in 2 mM Ca Ringer's should be 0.006 μmole per g of muscle. The total labeled calcium content in the center of the I band after only 5 min of soaking in ^{45}Ca Ringer's was at least 0.05 μmole per g of muscle. Calcium would have to be concentrated at least eight times within the transverse tubule to accommodate this amount.

Associated with excitation there is a release of intracellular calcium from the terminal cisternae (3–5). This calcium diffuses to the A band, initiates contraction, and is reaccumulated by the sarcoplasmic reticulum during relaxation (2–3). In the studies reported above, the calcium did not return directly to the terminal cisternae during relaxation from a tetanus. During the first minute of the recovery period, the intermediate cisternae and the longitudinal tubules of the reticulum contained more calcium and the terminal cisternae, less calcium than the same structures in either the unstimulated muscle or in the muscle 10 min after a tetanus. Since diffusion distances within the reticulum are small, the relatively high concentrations of calcium present in the longitudinal tubules and intermediate cisternae during the immediate post-tetanic period (Table III) indicate that some type of calcium binding was occurring. The intermediate cisternae and longitudinal tubules lost much of their calcium to the terminal cisternae over the next few minutes of the recovery period. The former structures were loaded with calcium, therefore, after the transient elevation in the sarcoplasmic concentration of calcium that occurred during contraction had been reversed by the action of a calcium-sequestering system in the reticulum (2, 13). Since the first parts of the reticulum to show an increase in calcium content after the end of a tetanus were the longitudinal tubules and the intermediate cisternae, it is likely that they contain the calcium-sequestering system that is believed to operate during relaxation. The calcium taken up by these structures presumably then moves more slowly to the terminal cisternae, the main storage site in the muscle which has completely recovered from mechanical activity. This scheme for activation and relaxation in some ways resembles the model proposed by Hodgkin and Horowicz (23).

The experimental results suggest that the amount of isotopic calcium in the intermediate cisternae and longitudinal tubules depends on the specific activity of the sarcoplasm. In resting muscles which had been soaked in ^{45}Ca

Ringer's solution for only 5 min and in which the specific activity of the sarcoplasm should have been very low, these structures contained little or no isotopic calcium in spite of a significant labeling of the terminal cisternae. This observation argues against diffusion within the reticulum from the terminal cisternae to the binding sites within the longitudinal tubules and the intermediate cisternae. The latter two structures contained a larger amount of calcium in muscles exposed to radioactive calcium for 5 hr at rest and in muscles previously stimulated in ^{45}Ca Ringer's. In the posttetanic state, which followed the period during which the specific activity of the sarcoplasm should have been the highest, they contained the most calcium. It appears that the binding sites for calcium in the intermediate cisternae and the longitudinal tubules are in equilibrium with sarcoplasmic calcium and differ from the sites in the terminal cisternae, which seem to be in equilibrium with calcium inside the reticulum. Since the binding sites in the terminal cisternae appear to be both different from those in the intermediate cisternae and longitudinal tubules and in equilibrium with a different pool of calcium, it is unlikely that calcium is released from the longitudinal tubules and intermediate cisternae as well as from the terminal cisternae following excitation of the muscle.

The largest amount of calcium found in the intermediate cisternae and the longitudinal tubules was at least 0.2 μmole per g of muscle (in the muscle frozen 30 sec after a tetanus at 4°C). The data of Weber and Herz (11) indicate that between 0.1 and 0.2 μmole of calcium per g of muscle is necessary to produce a maximal contraction. There seems therefore to be a sufficient number of binding sites in the reticulum in equilibrium with the sarcoplasm to produce relaxation without ion transport and to provide a potential mechanism for relaxation that would be more rapid than one dependent on ion transport. Ebashi (12) has pointed out that in order to produce relaxation the calcium pump would have to be much faster than the sodium pump. If the binding hypothesis is true, then the ATP used for the calcium pump should be hydrolyzed after relaxation. Kushmerick (17) has shown that a significant amount of ATP is split in the first few seconds after a muscle recovers from a tetanus. Also, consistent with a binding mechanism is the fact that the fastest rates of calcium uptake demonstrated with isolated reticulum are associated with the highest ratios of calcium transported per ATP split (15).

A relatively slow movement of calcium from the longitudinal tubules and the intermediate cisternae to the terminal cisternae during the recovery from a twitch would lead to the gradual depletion of calcium in the terminal cisternae during a series of twitches at a moderate frequency. This would produce a decrease in the degree of muscle activation. Since such a decrease has not been seen, it can be assumed that the movement of calcium within the reticulum during recovery from a twitch occurs very rapidly. The radioautographic data do not have adequate time resolution to measure the rate

of movement of calcium, but they do furnish sufficient information for one to make certain inferences. 20 sec after a tetanus at room temperature a significant amount of calcium that had been bound in the terminal cisternae in the fully recovered muscle was still bound in the longitudinal tubules and the intermediate cisternae, but there was no indication of how much calcium had already been transported from the longitudinal tubules and the intermediate cisternae to the terminal cisternae. As the low temperature studies indicate a capacity of at least 0.2 μmole per g of muscle in the longitudinal tubules and the intermediate cisternae, and since it is likely that at least 0.6 μmole per g is released during a tetanus (5) (Table II), the small amount of calcium still bound to the longitudinal tubules and the intermediate cisternae after 20 sec at room temperature was a minor fraction of what was initially bound. At least 0.1 μmole of calcium per g and possibly as much as 0.5–0.6 μmole per g had probably already been transported to the terminal cisternae in 20–30 sec after the tetanus at 25°C.

The radioautographic data suggest a possible mechanism for the phenomenon of posttetanic potentiation (21, 22). As the calcium content of the isolated sarcoplasmic reticulum increases, the rate of accumulation decreases (1, 16). If one assumes that the same phenomenon exists in the longitudinal tubules and the intermediate cisternae, then as their calcium content increases, the calcium still remaining in the sarcoplasm should act for a longer period of time. A twitch occurring when the calcium content of the intermediate cisternae and the longitudinal tubules is elevated should have a prolonged active state. The duration of the active state is longer (18) following a tetanus or even a brief series of stimuli. The increment in duration increases with the extent of the preceding activity. The association of posttetanic potentiation with the elevated calcium content in the longitudinal tubules and the intermediate cisternae is supported by the temporal correlation of the two phenomena in the studies reported above.

The intermediate cisternae and the longitudinal tubules of muscles exposed to ^{45}Ca Ringer's for only 5 min contained little or no isotope even when a significant exchange of calcium in the terminal cisternae had already occurred. If calcium is picked up from the sarcoplasm by the first two structures, then the calcium in the terminal cisternae must have exchanged with extracellular calcium by a route which did not involve the sarcoplasm. The exchange could have occurred directly between the transverse tubule, whose lumen is open to the extracellular space (14), and the terminal cisternae. About 80% of the transverse tubule is composed of a special junction with the terminal cisternae, and this could be the site involved in a direct exchange. The sarcoplasm would be labeled with ^{45}Ca as a result of a resting leakage or a stimulated release from the terminal cisternae.

The myosin-bound calcium exchanged slowly in the resting muscle and

much more rapidly in the contracting muscle. No clear understanding of the role of this calcium in the contractile process can be inferred from the above experiments and further study is indicated.

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