The Intracellular Site of
Calcium Activation of Contraction
in Frog Skeletal Muscle

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ABSTRACT   Radioautography has been used to localize ^{45}Ca in isotopically labeled frog skeletal muscle fibers which had been quickly frozen during a maintained tetanus, a declining tetanus, or during the period immediately following a tetanus or a contracture. During a tetanus almost all of the myofibrillar ^{45}Ca is localized in the region of the sarcomere occupied by the thin filaments. The amount varies with the tension being developed by the muscle. The movement of calcium within the reticulum from the tubular portion to the terminal cisternae during the posttetanic period has a half-time of about 9 sec at room temperature and a Q_{10} of about 1.7. Repolarization is not necessary for this movement. Evidence is given to support the notion that most calcium efflux from the cell occurs from the terminal cisternae into the transverse tubules.

INTRODUCTION

The mechanism by which the sudden increase in the concentration of calcium ions in the sarcoplasm following membrane depolarization produces a contraction involves a calcium-binding protein called troponin (Ebashi et al., 1967). Synthetic actomyosin, which does not contain troponin, does not specifically require calcium for contraction and hence is not uniquely triggered from the resting to the contracted state by an abrupt increase in calcium ion concentration (Weber and Herz, 1963). The addition of troponin and tropomyosin to the synthetic contractile system makes it calcium-sensitive. Troponin, rather than tropomyosin, is directly responsible for the calcium sensitivity, although both proteins are needed to demonstrate the effect. Troponin has a large capacity and a high affinity for binding calcium (Ebashi et al., 1967; Fuchs and Briggs, 1968). Almost all of the exchangeable calcium bound by the contractile proteins within the range of calcium concentrations where the transition between the relaxed and the contracted state occurs is bound to troponin.

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If these generalizations inferred from studies on subcellular systems are applicable to the intact cell, exchangeable calcium should be bound by troponin during a contraction, and some quantitative relation should exist between the amount of troponin-bound calcium and the extent of tension development. Biochemical and immunofluorescent studies suggest that in striated muscle troponin is relatively uniformly distributed along the thin filament (Ohtsuki et al., 1967, Hellam and Podolsky, 1969). One would expect, therefore, to find exchangeable calcium absent from the region of the thin filaments in resting muscle and present in significant amounts in contracting muscle. An earlier study of contracting muscles using radioautography showed that exchangeable calcium is restricted to the part of the thin filaments that extended into the A band (Winegrad, 1965). These experiments, however, involved a relatively primitive technique of activation and fixation. More reliable activation of the muscle and immobilization of cell calcium are possible with quick freezing and vapor fixation, and the studies reported in this paper deal with the application of the improved techniques to the localization of calcium in the contracting muscle. Calcium released from the terminal cisternae during muscle activation is not immediately restored to the terminal cisternae during relaxation. It is bound first by the tubular portion of the reticulum and then returned more slowly to the terminal cisternae (Winegrad, 1968). Additional experiments on muscles in the posttetanic state have been performed to clarify further the characteristics of these calcium movements.

METHODS

The general procedure used in these experiments has already been described (Winegrad, 1968). Bundles of 12–15 fibers from frog toe muscles were isolated and allowed to recover for 1 hr in Ringer's solution at room temperature. They were then soaked in ^{45}Ca-Ringer's solution for approximately 4–5 hr, during which time the specific activity of the exchangeable cell calcium rose to over 50% of that in the soak solution. Some ^{45}Ca-labeled muscles were transferred to the quick freeze apparatus and tetanized by stimuli from electrodes in contact with each end of the muscle. 2 msec pulses at 1.5 threshold voltage and a frequency of 50/sec were used. The tissues were frozen either during the tetanus plateau, during the declining phase of a prolonged tetanus, or 2 sec after the end of a tetanus. Some muscles were cooled in ice jackets during the last 15 min of the soak in ^{45}Ca-Ringer's solution before stimulation, but the results of the experiments were not significantly altered by this cooling.

A second group of muscles was transferred from regular ^{45}Ca-Ringer's solution to precooled (4°C) ^{45}Ca-Ringer's in which all of the NaCl had been replaced by choline chloride and to which tubocurarine had been added to block any pharmacological action of the large concentration of choline. This substitution was made to prevent the initiation of action potentials during subsequent procedures. After 15 min in isotopic choline-Ringer's solution, the muscles were exposed for 25 sec to either choline-^{45}Ca-Ringer's containing 100 mM KCl or ^{45}Ca-Ringer's in which all

of the NaCl had been replaced by KCl. Complete relaxation from the contracture produced by the high potassium occurred during the 25 sec. The muscles were then quickly dipped into nonradioactive high K$^+$ solution to wash off the adherent radioactive solution and quickly frozen 30 sec after the initial exposure to high K$^+$.

A third set of muscles was washed out in five different tubes of nonradioactive Ringer's solution for a total of 10 min before being quickly frozen.

All muscles were vacuum dried at −60°C, fixed in osmium vapor, and embedded in methacrylate. Radioautographs were made from thin sections of each muscle in a manner which has already been described (Winegrad, 1965). The amount of calcium present in various parts of the cell was estimated from radioautographs as previously described (Winegrad, 1968).

RESULTS

The method of presenting data is illustrated in Fig. 1 A, which describes radioautographs of a toe muscle that was fixed during tetanic stimulation at 50/sec while tetanus tension was being well maintained. The solid line is based on the localization of all grains in the radioautographs and indicates the relative grain density over different parts of the sarcomere, including both the myofibrils and the space between the myofibrils. The unshaded and shaded portions of the area under the solid line represent the relative concentrations of ^{45}Ca in the various parts of the myofibrils and the interfibrillar space, respectively. This transverse distribution was estimated in the following way. The grain densities over the central third of the myofibril and over the space between the myofibril in each of the longitudinal zones of the sarcomere were measured, and the count over the central third of the myofibril was multiplied by 2.8, the ratio of myofibril to interfibrillar space in the muscle sections. The ratio of this product to the grain density over the interfibrillar space is indicated by the ratio of unshaded to shaded area at any given position along the sarcomere. Although this method is a reasonable approximation of the relative amounts of calcium in the myofibril and in the reticulum, it somewhat overestimates the relative amount of calcium between the myofibrils because of the spread of grains in the emulsion around the ^{45}Ca in the muscle section (Winegrad, 1965). The production of grains over the interfibrillar space by ^{45}Ca in the myofibril will be greater than the production of grains over the central third of the myofibril by ^{45}Ca in the interfibrillar space because of the geometry in which the interaction of the ^{45}Ca β-particles with the photographic emulsion occurs (Winegrad, 1965).

Although the majority of grains in radioautographs of the muscle fixed during the plateau of tetanus tension were over the space between the myofibrils and due presumably to ^{45}Ca in the reticulum, a considerable number of grains were over two parts of the myofibril, the I band and the portion of the A band where the thin and thick filaments overlap. This grain distribution corresponds to ^{45}Ca distributed primarily in the tubular portions of the sarcoplasmic reticulum and in the region of the thin filaments. A small
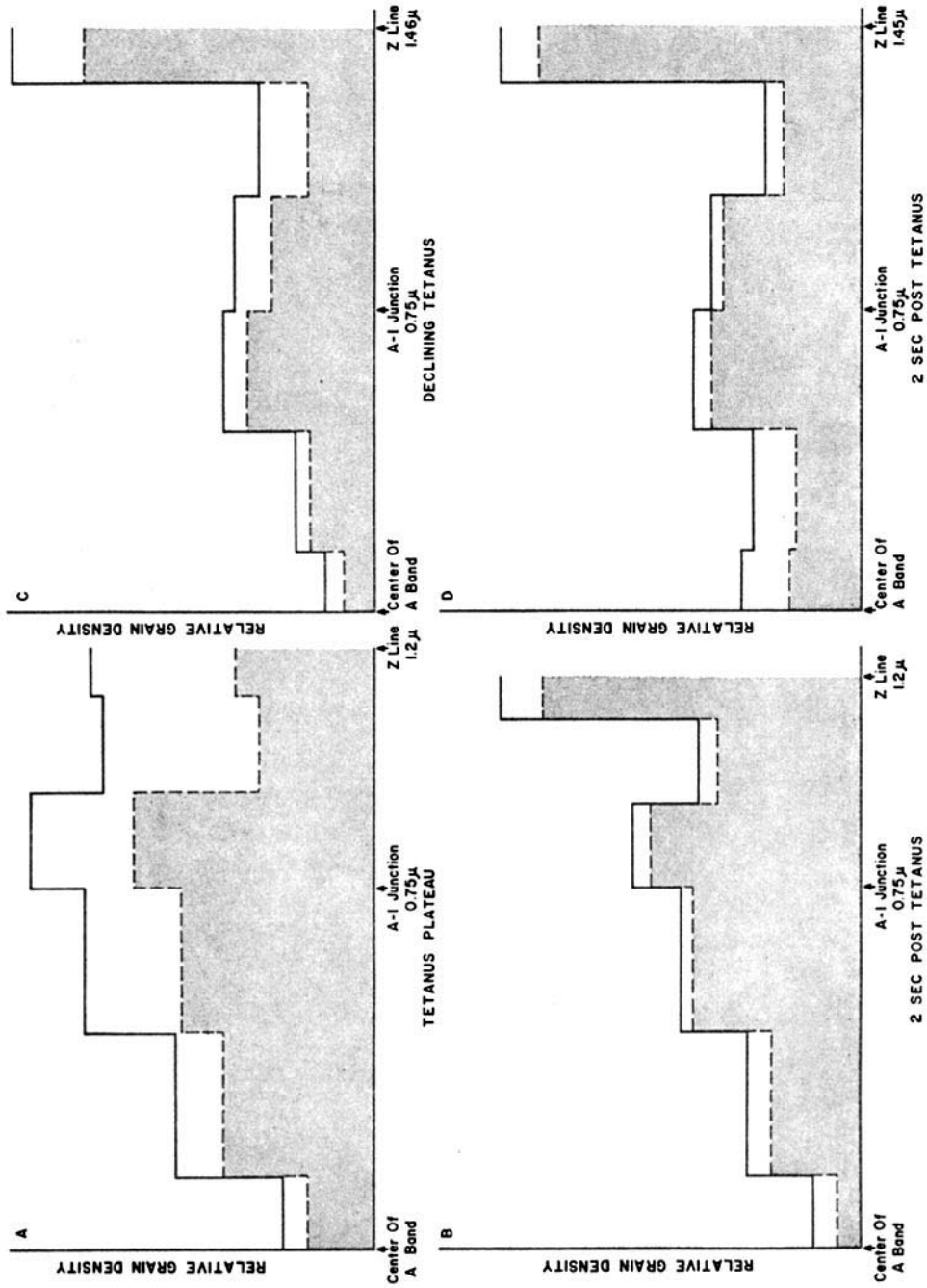
FIGURE 1. The relative grain densities in radioautographs of ^{45}Ca-labeled muscles frozen at the following times: (A) during a maintained tetanus, (B) 2 sec after the end of a maintained tetanus, (C) during a declining tetanus, and (D) 2 sec after the end of a declining tetanus. For explanation, see the text.

amount of ^{45}Ca was present in the region of the triads, presumably in association with the terminal cisternae of the sarcoplasmic reticulum. About one-third of the approximately 0.6 μmole of calcium/g of muscle which had exchanged with the extracellular ^{45}Ca was located in the region of the thin filaments.

When a toe muscle was stimulated at 50/sec for more than a few seconds, the tetanus tension began to fall. Fig. 1 C shows the distribution of grains in radioautographs of a toe muscle which had been tetanized long enough for tension to have fallen to approximately 40% of maximum. The relative amounts of ^{45}Ca in the myofibrils and in the reticulum are again indicated by the unshaded and shaded areas. The distribution of ^{45}Ca in this muscle differed from that in the muscle frozen during the plateau of tetanus in two major ways. The region of the thin filaments contained much less calcium than the analogous region in a muscle frozen while still generating maximum tetanus tension. In addition, the region of the terminal cisternae in the muscle with the weaker tension contained more ^{45}Ca.

2 sec after the end of a tetanus, the ^{45}Ca distribution had changed considerably from what existed during the tetanus. In a muscle which had maintained maximum tetanus tension throughout the entire period of stimulation, almost all of the ^{45}Ca was localized between the myofibrils, and the small amount of ^{45}Ca in the myofibrils was somewhat more concentrated in the A band and the center of the I band (Fig. 1 B). The ^{45}Ca between the myofibrils was localized in both the tubular region and the terminal cisternae of the sarcoplasmic reticulum. About two-thirds of the total ^{45}Ca in the muscle was in the tubular portion of the reticulum, and most of the rest was in the terminal cisternae. A comparison between a muscle fixed during the plateau of a tetanus and one fixed 2 sec after the end of such a tetanus shows that about the same amount of ^{45}Ca was present on the tubular portion of the reticulum in both muscles. The decrease in myofibrillar ^{45}Ca was almost equal to the increase in ^{45}Ca in the terminal cisternae. In the 2 sec after a tetanus that had fallen about 60% during the stimulation, a similar shift in ^{45}Ca occurred even though the amount of ^{45}Ca in the myofibril of the contracting muscle was less (Fig. 1 D). 1 min after the end of a tetanus, most of the ^{45}Ca in the tubular portion of the reticulum shifted to the terminal cisternae (Fig. 2). No significant difference in the ^{45}Ca distribution 1 min after a tetanus existed between the muscles which had been stimulated for the longer and the shorter periods.

Radioautographs were also prepared from muscles which, though still depolarized from high extracellular K$^+$, had completely relaxed from a K$^+$ contracture. The purpose was to determine the effect of maintained depolarization on the restoration of ^{45}Ca to its resting location (Fig. 3). Almost all of the tissue ^{45}Ca was present between the myofibrils, and the distribution
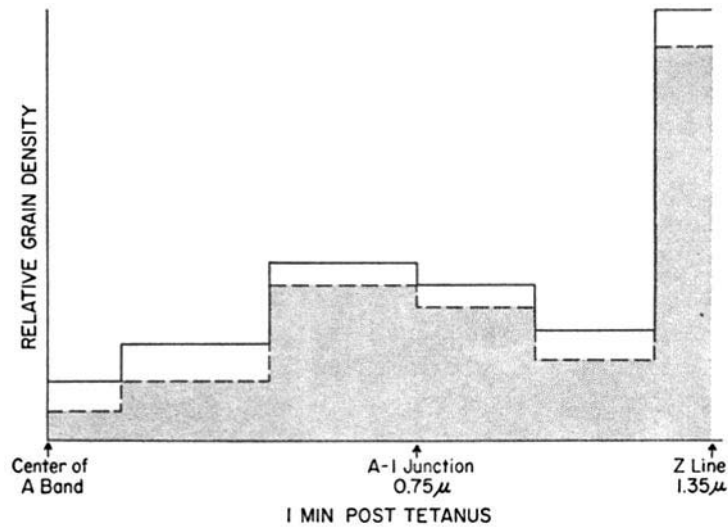
FIGURE 2. The relative grain density in radioautographs of a ^{45}Ca-labeled muscle frozen 1 min after the end of a tetanus.
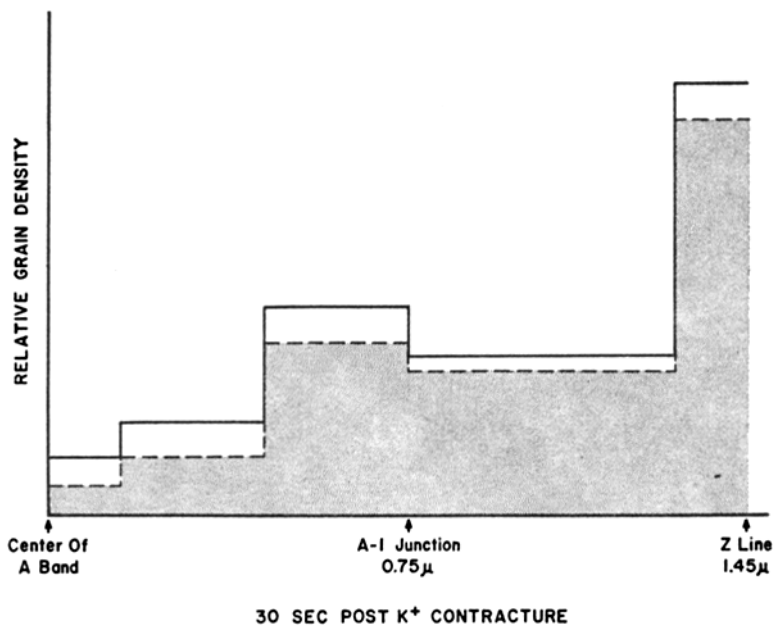

FIGURE 3. The relative grain density in radioautographs of a ^{45}Ca-labeled muscle frozen 30 sec after the initial exposure to high K$^+$-Ringer's solution. The muscle had completely relaxed from the contracture but still remained depolarized in the high K$^+$ solution.

within this space resembled that in the muscles frozen 1 min after the end of a tetanus. These results indicate that restoration of the normal resting potential is not necessary for the return of the ^{45}Ca to its resting location in the reticulum.

In all of the studies of muscles which had relaxed from tetanus or contracture, there was, as has been previously noted, a small localization of grains in the A band portion of the myofibril. This focus of ^{45}Ca was not always clear in muscles frozen during a tetanus, but it may have been obscured by the sizable amounts of ^{45}Ca present in the I band portion of the thin filament and in the reticulum in the more central parts of the sarcomere.
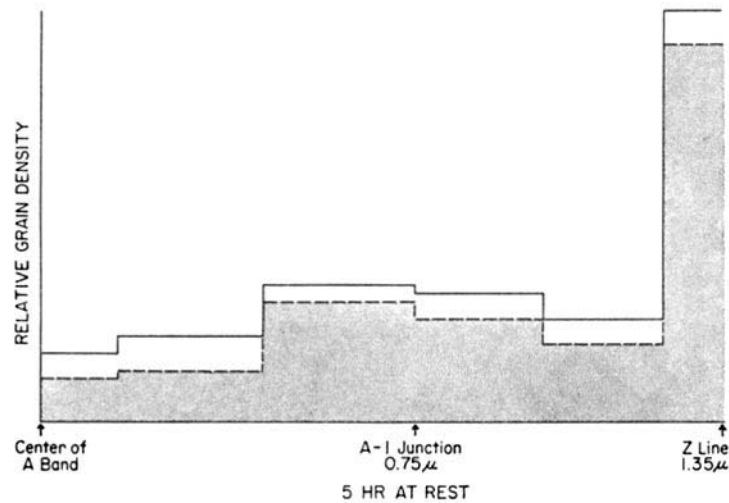

FIGURE 4. The relative grain density in radioautographs of a ^{45}Ca-labeled muscle frozen at rest after a 5 hr soak in ^{45}Ca-Ringer's solution.

In an earlier study (Winegrad, 1968), the first calcium in the cell to exchange with extracellular ^{45}Ca was in the region of the triads. The amount seemed to be too large for just the transverse tubules alone, and the calcium exchange was assumed to involve the terminal cisternae. A substantial delay occurred in the resting muscle, however, before the tubular portion of the reticulum was isotopically labeled. These data were interpreted as indicating that most of the ^{45}Ca entered the cell by crossing from the lumen of the transverse tubule directly into the terminal cisternae. The opposite type of experiment was performed to see if the major pathway of calcium extrusion from the cell was from the terminal cisternae to the transverse tubules. Muscles labeled with ^{45}Ca for 5 hr were soaked in nonradioactive solution for 10 min and then frozen. The major difference between the localization of ^{45}Ca in these muscles and in those that had not been washed out in non-

radioactive solution (Fig. 4) is the relatively smaller amount of ^{45}Ca between the myofibrils in the center of the I band (Fig. 5). These data would indicate that most of the calcium efflux from the cell during the 10 min washout occurred from the terminal cisternae.

DISCUSSION

The location of exchangeable calcium in resting, tetanized, and posttetanic frog skeletal muscle has been investigated with quick freezing and vapor
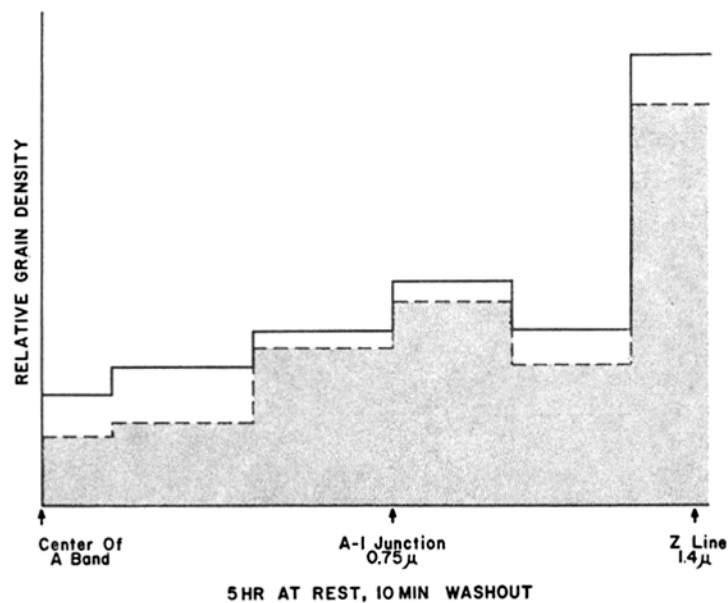

5 HR AT REST, 10 MIN WASHOUT

FIGURE 5. The relative grain density in radioautographs of a ^{45}Ca-labeled muscle frozen after a 10 min washout in nonradioactive Ringer's solution following a 5 hr soak in ^{45}Ca-Ringer's solution.

fixation to minimize any translocation of tissue ^{45}Ca. Both the well-maintained tetanus and the declining tetanus were studied to evaluate any quantitative correlation between the tension and calcium localization. The analysis was facilitated by exposing the experimental muscles to ^{45}Ca long enough for the specific activity of the reticulum calcium to have reached approximately that of the soak solution.

During the peak of tetanus about 0.2 μmole of calcium/g was localized in the region of the thin filaments, and most of the remaining 0.4–0.5 μmole/g was present in the tubular portion of the longitudinal reticulum. There was a small amount localized in the terminal cisternae of the sarcoplasmic reticulum. By contrast, resting muscle contained practically no ^{45}Ca in the I

band portion of the myofibril, a small amount in the tubular portion of the reticulum, and a very large concentration in the terminal cisternae. During tetanus, therefore, most of the calcium in the terminal cisternae is shifted to the region of the thin filaments and the tubular portion of the reticulum. Experiments with muscle models have demonstrated that a binding by the myofibrils equivalent to about 0.10–0.15 μmole of calcium/g of muscle is necessary for maximum activation of contraction (Weber and Herz, 1963). Since the thin filaments of maximally contracting muscle fibers bound about 0.2 μmole of calcium/g of muscle, it appears that the calcium requirement for contraction exists in some component of the thin filament. Most of the calcium within the thin filaments is probably bound to troponin, the calcium-binding protein which is distributed uniformly along the thin filaments (Ohtsuki et al., 1967). The calcium content of the terminal cisternae was greatly reduced during the period of maintained tetanic tension, and it is likely, therefore, that during this period each stimulus releases only a small amount of calcium to the sarcoplasm. The total loss of calcium from the terminal cisternae was equal to the calcium in the myofibril plus some in the tubular portion of the reticulum.

When the period of tetanic stimulation was prolonged, tetanus tension began to fall. Associated with the decline in tension in these muscles were an increase in the content of calcium in the terminal cisternae and a decrease in the amount of calcium in the vicinity of the thin filaments. It appears that the smaller action potential that was produced during the declining tetanus[1] was less effective in releasing calcium from the terminal cisternae, but that the process involved in the return of calcium to the terminal cisternae was not affected.

About half as much calcium was present in the thin filaments of muscles in which the tetanic tension had declined to 40% of the peak level as there was in muscles contracting maximally. This relationship between thin filament calcium and tension is consistent with the much more rigorously determined relationship between calcium content of isolated myofibrils and extent of superprecipitation, which is the analogue of contraction in this system (Weber and Herz, 1963). The presence of calcium along the entire length of the thin filaments in contracting muscles differs from earlier results that had indicated a localization only where thick filaments overlapped thin filaments (Winegrad, 1965). The earlier studies, however, involved chemical fixation in aqueous and alcoholic solutions, which are known to produce different effects on the overlapped and nonoverlapped portions of the thin filaments (Page and Huxley, 1963). Furthermore, the tissues that were developing tension during fixation with osmium solution lost calcium in

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[1] Connolly, R., J. Spear, and S. Winegrad. Unpublished results.

amounts relatively similar to the calcium content of the nonoverlapped portions of the thin filaments in contracting muscles as measured above (Winegrad, 1965).

From these and previously published data (Winegrad, 1968) it is possible to estimate grossly the kinetics and the temperature dependence of the movement of calcium from the tubular reticulum to the terminal cisternae. At room temperature 2 sec after the end of a sustained tetanus, the tubular portion of the reticulum contained about 0.4 μmole of calcium/g of muscle, and 18 sec later it contained about 0.1 μmole. If movements follow first-order kinetics, then at room temperature the half-time is about 9 sec. When the experiments were conducted at 4°C, the tubular reticulum contained about 0.4 μmole and 0.2 μmole of calcium 2 sec and 30 sec, respectively, after a tetanus, indicating a half-time for the movement within the reticulum of about 28 sec. The Q_{10} for the process is therefore about 1.7. Weber et al. (1966) concluded that the Q_{10} for the rate of calcium uptake by isolated microsomes was 2.5–3.0. Since the movement of calcium from the tubular reticulum to the terminal cisternae does not involve a process with a high activation energy, the splitting of ATP associated with the active accumulation of calcium by the reticulum may occur during the initial binding of calcium by the reticulum. This inference is consistent with the evidence that relaxation of muscle, which is presumably produced by the initial binding of calcium by the reticulum, has a Q_{10} of about 3 (Jewell and Wilkie, 1960). Repolarization of the surface membrane is not necessary for the restoration of calcium to the terminal cisternae, as continued exposure to high K^+ after a K^+ contracture is without effect.

When this recovery movement of calcium within the reticulum was first observed (Winegrad, 1968), it was tentatively linked with the classical post-tetanic potentiation that lasts for several minutes following a tetanus (Gutt-man et al., 1937). It is now clear that the temporal correlation between the two events is poor, because at room temperature the calcium movements are essentially over in 30 sec. The characteristics of the twitch in the first 10–20 sec after a tetanus have been examined, however, and during this period the velocity of contraction gradually increases and the duration of contraction gradually decreases.[2] Both phenomena occur at the same rate. The changes in velocity and duration of contraction are consistent with a depletion of calcium in the terminal cisternae, depressing activation, and an accumulation in the tubular reticulum, slowing the rate of further calcium uptake (Makinose and Hasselbach, 1965).

The more rapid loss of ^{45}Ca from the terminal cisternae than from the tubular portion of the reticulum in resting muscles supports the hypothesis

[2] Connolly, R., and S. Winegrad. Unpublished data.

that a major portion of calcium efflux takes place from the terminal cisternae to the transverse tubular lumen. The exchange of calcium between the terminal cisternae and the transverse tubules, however, is much slower than the fastest rate of movement of calcium from the tubular reticulum to the cisternae. Therefore the decline in the concentration of isotope in the tubular portion of the reticulum is probably determined by the rate of leakage of ^{40}Ca from the terminal cisternae to the sarcoplasm. In 10 min the concentration of ^{45}Ca in the terminal cisternae fell about 30%, and that of the tubular reticulum about 10%. Since the total pool of exchangeable sarcoplasmic calcium is about 0.4 μmole/g muscle (0.3 μmole myofibril + 0.1 μmole reticulum) (Winegrad, 1968), the tubular transport of calcium should approximately equal 0.4 × 0.1 × (100/15) × (1/600), or 0.4 × 10^{-9} mole/ sec/g of muscle. Weber et al. (1966) found the rate of leakage from isolated reticulum to be about 2 × 10^{-9} mole/sec for the reticulum contained in 1 g of muscle.

The small localization of calcium in the A band portion of the myofibrils noticed earlier (Winegrad, 1968) has been observed again. Unlike the results of previous studies, however, there is no indication that the isotope is absent from that part of the A band which contains thin filaments. The significance of this exchangeable calcium is not clear, particularly in view of the observations of Fuchs and Briggs (1968) and Ebashi et al. (1967) that myosin does not bind calcium at the intracellular concentrations of free calcium that must exist in the resting muscle.

The data presented above are compatible with a model for intracellular calcium movement that was proposed in a previous paper (Winegrad, 1968). The model states that most of the exchange of intracellular calcium with extracellular calcium takes place between the terminal cisternae and the lumen of the transverse tubules. This is a more efficient mechanism than efflux across the surface membrane, because the electrochemical gradient opposing the latter is probably about 10^3 times greater than that opposing the former (Carvalho and Leo, 1967). The calcium in the sarcoplasm, the tubular portion of the reticulum, and the contractile filaments exchanges with calcium leaking from the terminal cisternae. This leak is small in the resting muscle but is greatly increased by stimulation. Calcium returns to the terminal cisternae from the sarcoplasm after it has been bound by the tubular reticulum and actively transported into the lumen of the reticulum.

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