CALCIUM INFLUX IN SKELETAL MUSCLE AT REST, DURING ACTIVITY, AND DURING POTASSIUM CONTRACTURE

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ABSTRACT

Calcium influx in the sartorius muscle of the frog (*Rana pipiens*) has been estimated from the rate of entry of Ca^{45}. In the unstimulated preparation it is about equal to what has been reported for squid giant axons, but that per impulse is at least 30 times greater than in nerve fibers. The enhanced twitch when NO_3^- replaces Cl^- in Ringer's is associated with at least a 60 per cent increase in influx during activity, whereas this anion substitution does not affect the passive influx significantly. Calcium entry during potassium contracture is even more markedly augmented than during electrical stimulation, but only at the beginning of the contracture; thus, when a brief Ca^{45} exposure precedes excess K^+ application, Ca^{45} uptake is increased three- to fivefold over the controls not subjected to K^+, whereas when Ca^{45} and K^+ are added together, no measurable increase in Ca^{45} uptake occurs. These findings are in keeping with the brevity of potassium contracture in "fast (twitch)" fibers such as in sartorius muscle.

Considerable data have accumulated of an indirect nature suggesting that the entry of calcium into the muscle fiber may be a link between the action potential (or other depolarization) and contraction (or contracture) (32). Earlier studies showed no measurable change in the calcium content of stimulated muscle (5). However, the absence of such a change does not necessarily constitute evidence against the entry of calcium. For such entry may occur but be obscured by subsequent increase in the rate of exit. Or the entry may be too small to be detected by usual techniques and yet be large enough to initiate contraction. Thus, calcium is the only physiological ion which, when injected at low concentrations into muscle fibers, causes shortening (11); this is consistent with the very low thermodynamic activity of the calcium normally present in protoplasm, apparently because of binding (*e.g.* 3, 9, 14), hence the

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entry of a small amount of calcium may represent at least a transitory, large relative change in the amount of ionized calcium present.

The studies to be described show that the two possibilities are quite real. Moreover, it will be seen that the augmentation in twitch height resulting from replacement of the chloride in the medium with nitrate (18, 19) is accompanied by a proportionately equal increase in calcium entry, but only during activity. Also, the time course of calcium entry corresponds to that of potassium contracture. Finally, it will be shown that the characteristics of influx in contracting muscle are sufficiently different from those of active nerve fibers as to suggest a special mechanism related to contractility.

Methods

The familiar technique of measuring the uptake of Ca^{45} was used to determine influx, but two important modifications were employed routinely to increase the sensitivity of the method so that small experimental differences such as we discovered were involved could be measured.

First of all, the exposure to "hot" Ringer's was usually kept short, namely, 10 minutes. During the second half of this interval the experimental condition of supramaximal stimulation was introduced for one of a pair of muscles while the mate served as the unstimulated control. Two purposes were served by the shortness of the exposure: (*a*) The passive uptake of Ca^{45} by the fibers was kept small so that it did not obscure an equally small increment in Ca^{45} uptake brought about by the experiment, and (*b*) an appreciable backflux did not develop by virtue of a substantial rise in intracellular Ca^{45}, which otherwise would have reduced our estimates of the rate of entry. It will soon be shown that 60 minutes is too long because of the latter; also, that 10 minutes is not so short as to limit fiber entry appreciably by virtue of incomplete diffusion in the extracellular space—already noted by Harris in his studies of anion penetration (10). Second, before the tissues were dried and ashed for counting, the Ca^{45} in the extracellular water and on the superficial binding sites of the surface of the fibers and in the connective tissue was removed routinely by soaking the individual muscles at frequent intervals (5, 10, 25, and 50 minutes) in a series of radioisotope-free Ringer's, each of 5 ml., for a total of 90 minutes. This removes most of the extraneous Ca^{45} (34), which would have obscured the changes in the Ca^{45} content of the myoplasm. An upward correction of only 10 per cent was necessary for the loss of Ca^{45} from the fibers themselves (34).

The special conditions employed for the study of potassium contracture will be given with the results.

Glass units, essentially as previously described (30), but without electrodes, served to circulate and oxygenate 3 ml. of normal Ringer's solution (1.0 mM/liter Ca^{++}, 1.6 mM/liter K^+, all-sodium Sørensen phosphate buffer with the osmolarity of 1 mM/liter NaCl, plus NaCl bringing the total molarity to 0.111) containing Ca^{45} at a level of 1 microcurie.[1] A low concentration of cocaine, 2 mg. per cent, was invariably

[1] The radiocalcium was added to Ringer's from a stock of neutralized, high specific activity (*ca.* 300 mc./gm.) $CaCl_2$ solution obtained from the Oak Ridge National Laboratory and designated CA-45-P-3.

present to prevent spontaneous twitching. In NO_3^- Ringer's only 75 per cent of the Cl^- was replaced by NO_3^-.

Each of a pair of sartorii from the same animal (*Rana pipiens*) was mounted in its own unit. The distal end was tied to the uninsulated end of an insulated heavy copper wire that ran to the bottom of the column of circulated solution; the wire served as a support as well as one of the stimulating electrodes. The proximal end of the muscle was attached to the isotonic, ink-writing lever used to follow the contractions. The second stimulating electrode was identical to the first but made contact with the top of the column of circulating solution. Thus, the stimulating current (usually a 5 msec. rectangular pulse delivered with a Tektonix generator) flowed parallel to the muscle. The electrodes did not touch the fibers. The stimulus was adjusted to two or three times above maximal strength. That the flow of current was not itself responsible for the uptake of calcium was shown by observing the absence of a change in the Ca^{45} uptake when stimulating currents were increased in duration threefold.

Stimulation was usually at a rate of one shock per second, for 5 minutes or less depending on the ability of the muscles to respond adequately. This somewhat less than doubled the radioactivity taken up compared to that in the unstimulated control. In some experiments a rate of one shock every 2 seconds was employed; these gave the same influx per twitch as the higher rate, but with less accuracy because of the smaller increment of radioactivity over that of the controls.

Small experimental effects were usually determined by the use of paired preparations. Thus, the additional uptake with activity was measured relative to unstimulated controls; that in nitrate Ringer's was determined relative to similarly stimulated controls in chloride Ringer's.

The muscles were weighed at the end of the experiments, dried at 105°C. and reweighed to determine the water content, and then ashed for at least 10 hours at 500°C. The residue was shaken for 4 hours in 0.1 N HCl and duplicate samples of this extract deposited and dried on planchets as previously described (33). Diluted samples of the original hot soak solutions were also counted, for this activity and the known calcium content of the medium provided a convenient means of converting the Ca^{45} taken up by the fibers to micromoles of calcium actually transferred. As already mentioned, this Ca^{45} uptake was corrected for the 10 per cent loss during washout.

An estimate of surface area was also needed to compute the fluxes. If the average fiber diameter is taken as 100 micra, and the fibers constitute a fraction of the total muscle given by the ratio: (total water − extracellular water)/(total water) (=65/80 or 0.8), one obtains 300 cm.²/gm. The average fiber diameter probably lies between 80 and 100 micra (31), corresponding to 500 and 300 cm.²/gm. The latter figure has been employed in our estimates of flux.[2]

The experiments were all carried out at a room temperature of 25°C.

RESULTS

The influx data and their standard errors are all tabulated in Table I.

Muscle at Rest.—The influx estimated from the uptake of Ca^{45} for 60 minutes is probably significantly smaller than that estimated from the uptake in 10

[2] The average fiber diameter in frog (*R. temporaria*) sartorii is given as 102 micra (Carey, M. J., and Conway, E. J., *J. Physiol.*, 1954, **125**, 232).

minutes ($P = 0.1$). As pointed out above, the longer the exposure to Ca^{45}, the greater the deviation of uptake from linearity to be expected because of back-flux of the accumulating radioisotope; hence the smaller the estimated influx will be. The difference found is about 50 per cent larger than would be due to this factor alone, for the time constant of Ca^{45} uptake, estimated from efflux curves after 4 hours of Ca^{45} uptake (34), is in the neighborhood of 500 minutes; from this, the 60 minute influx would be expected to be 12 per cent smaller, or one-half of that indicated by the data. The greater difference is attributable to random variation, especially since the two sets of figures were not obtained with paired muscles.

TABLE I

Calcium Influx in Frog Sartorius Muscle

No. of experiments	Conditions	Influx	(B − A)*
	(Unstimulated)	*μμmole/cm.² sec.*	*μμmole/cm². sec.*
6	60 min. in Ringer's (A)	0.072 ± 0.0093	
6	60 min. in Ringer's + 20 mM KCl (B)		0.052 ± 0.011
23	10 min. in Ringer's	0.094 ± 0.0066	
12	10 min. in NO_3^- Ringer's	0.108 ± 0.018	
14	10 min. in tripled Ca Ringer's	0.34 ± 0.022	
	(Maximal twitches at 1/sec. or 0.5/sec.)	*μμmole/cm.² twitch*	*μμmole/cm.² twitch*
9	5 min. rest, 5 min. stimulation in Cl^- Ringer's (A)	0.20 ± 0.041	
12	5 min. rest, 5 min. stimulation in NO_3^- Ringer's (B)		0.12 ± 0.050

* The differences and standard errors are based on paired preparations to assure adequate precision. $P < 0.03$ or better for all data, hence the *changes* in influx as well as the influxes are significant.

The muscles used to measure the 60 minute uptake in Ringer's were compared with their mates subjected for the same period to a Ringer's containing an *additional* 20 mM/liter KCl. The increment is seen to represent about three-fourths of the control influx. Although an increase was also seen with short exposures to such KCl Ringer's, variability was greater and hence the data less satisfactory. All other experiments are based on short exposures to Ringer's containing radiocalcium, the usual period being 10 minutes except for the contracture experiments described below.

Paired muscles, in solutions differing only in that $NaNO_3$ rather than NaCl was used to make up the Ringer's, showed no significant difference in influx. The NO_3^- data from these and other experiments, averaged in Table I, are not significantly different from the Cl^- results.

Ca^{45} influx is increased approximately in proportion to the threefold increase in the calcium content of the medium. The uptake in a nitrate Ringer's equally high in calcium was not significantly different; therefore, all these data were averaged together in Table I.

Stimulated Muscle.—The increment in influx, obtained by comparing stimulated and unstimulated paired muscles, when divided by the number of twitches, gives the influx per twitch; the figure for this influx is about double that of the resting influx. If the calcium entry occurs only during the spike, which is about 1 msec. long, then the entry per twitch represents a rate of the order of 2000-fold of that at rest; or if entry takes place during the negative after-potential as well, which has a time constant of 13.5 msec. (5), the rate is still of the order of 100 times that at rest. Although there is no evidence against the greater entry occurring in part after the action potential, this is regarded as unlikely.

The increment in influx, when nitrate replaces chloride as the major anion of Ringer's, is seen to be 60 per cent. Since the resting influx is unaltered by nitrate, the increase is attributable completely to the activity. This finding therefore validates the prediction, on theoretical grounds, that nitrate would be found to increase the entry of calcium during muscle activity (32).

The 60 per cent figure for the increase in the influx of activity in nitrate may be somewhat on the low side, for the contractility of stimulated, nitrate-treated muscles tended to decline sooner than that of the controls (*cf.* reference 19). Our data do not permit a statement as to whether the decline of contractility is due to fewer functional fibers or to lowered effectiveness of the contractile mechanism. Preliminary observations of extracellular recorded action potentials suggest the former. Moreover, Sandow (27) has shown that the excitable mechanism fails before the contractile. Hence, a correction may be applied based on the ratio of the actual area under the contraction-time curves to the area that would have been obtained if the twitches had shown no decline. On this basis, nitrate increases activity influx 77 per cent.

An examination of the areas under the twitch height—time curves obtained with isotonic levers reveals an unanticipated direct relationship between the strength of the twitch and the amount of calcium entering. Thus, since the time of stimulation was the same for muscles in NO_3^- or Cl^- Ringer's, the areas are proportional to the magnitude of lever displacement or to the work done in deflecting the lever system. The uncorrected areas for twelve pairs of muscles were 39.4 ± 2.0 cm.^{2} in NO_3^- and 24.8 ± 0.95 cm.^{2} in Cl^-, or an increase of 60 per cent in NO_3^-. This is exactly the percentage increase in the entry of calcium—0.12/0.20 or 60 per cent. Such exact agreement suggests a simple, direct relation between the calcium entering and twitch height.

If, as above, the decline in the height of contraction is attributed to fewer active fibers, then the correction factor based on the areas of the twitch height–time curves

for calcium influx of muscles in chloride Ringer's is 8 per cent, making the influx/twitch 0.22 $\mu\mu$M/cm.^{2}, and for muscles in nitrate Ringer's it is 23 per cent, making the influx/twitch 0.39 $\mu\mu$M/cm.^{2}, or 77 per cent greater than that in chloride Ringer's, as given above.

Preliminary experiments suggested that threefold elevation of the extracellular calcium content delays the decline of the contractile response with repetitive activity in both nitrate and chloride Ringer's. Consequently, the uptake of Ca^{45} with stimulation was checked in these solutions in a few experiments. It was immediately apparent that the threefold increase of resting influx described above was not accompanied by a corresponding increase in the influx of activity, for neither the difference on stimulation in nitrate nor the increase with stimulation itself was large enough to be measurable in the presence of the large passive increase.

Potassium Contracture.—The increased entry of calcium in fibers depolarized by 20 mM/liter potassium is small although in the direction to be expected from enhanced ion pair formation (see Discussion). The small magnitude may be due to the depolarization being less than a threshold value needed for a high rate of calcium entry. This is suggested by the finding that potassium contracture is appreciable only at still higher potassium concentrations (13, 27). Consequently, the experiments were repeated at higher potassium concentrations. For this purpose, half of the sodium in the Ringer's was replaced with potassium and an additional 50 mM/liter of potassium salt was added in excess so that the solution was 1.5 times hypertonic. Thus, the tendency of the muscle to swell by virtue of replacement of sodium with potassium is counteracted for short times by the hypertonicity, for water movement is faster than that of KCl (29).

Two other aspects of potassium contracture were taken into account in the design of our experiments, namely, that contracture is brief despite the continued presence of potassium (13, 27) and that it is larger and more prolonged when NO_3^- replaces Cl^- (27). On the assumption that the limited duration is due to the enhanced Ca^{++} entry being equally brief—subsequently confirmed—the period of the experiment was kept short so that passive entry would not mask the effect. And to obtain longer contractures (and calcium entry?), the experiments were carried out in NO_3^- Ringer's, the excess potassium being added as KNO_3.

The contractures, recorded during the course of all experiments, were practically at an end in 1.5 to 2.5 minutes. Consequently, the intervals for the two experimental series were as follows: In the first series the experimental muscles were transferred from non-radioactive chloride Ringer's to hot, high potassium, nitrate Ringer's until the contracture was ended, then washed as usual for 90 minutes in "cold" chloride Ringer's and ashed. The controls were treated similarly, except that they were soaked for the same time in hot nitrate

Ringer's. In the second series the experimental muscles were first transferred for 2.5 minutes to hot nitrate Ringer's and then, for the duration of the contracture, to hot, high potassium, nitrate Ringer's; the controls remained in hot nitrate Ringer's for the same total period.

The data are shown in Table II. When Ca^{45} and high potassium are added together, there is a negligible effect of depolarization on Ca^{45} uptake. But when Ca^{45} is given slightly before, there is a three- to sixfold increase in Ca^{45} entry associated with the contracture. The passive uptake in the controls cor-

TABLE II

Calcium Uptake, Measured by Ca^{45} Entry, During Potassium Contracture

Conditions	Ca^{45} exposure	Contracture duration	Calcium uptake		$X - C$
			X	C	
	sec.	*sec.*	*μM/gm. × 10³*		*μM/gm. × 10³*
0.1 M/liter KNO_3, in Ringer's containing	90	75	6.5	11.1	−4.6
0.05/liter $NaNO_3$, added simultaneously with Ca^{45} (X) compared with	125	110	6.5	7.5	−1.0
	150	135	5.4	6.5	−1.1
equal Ca^{45} exposure in 0.111 M/liter $NaNO_3$ Ringer's (C)	120	105	9.9	8.4	+1.5
	Average				−1.3
0.1 M/liter KNO_3, in Ca^{45} Ringer's containing 0.05 M/liter $NaNO_3$, preceded	280	120	10.8	3.0	7.8
	275	90	15.0	3.4	11.6
ca. 2.5 min. by Ca^{45} in 0.111 M/liter	265	90	20.1	3.5	16.6
$NaNO_3$ Ringer's (X) compared with	280	120	22.7	6.4	16.3
equal total Ca^{45} exposure in 0.111 M/liter $NaNO_3$ Ringer's (C)					
	Average				13.1

responds to an influx of 0.05 μμM/cm.²sec., or about half of that obtained with 10 minutes' exposure to Ca^{45} (Table I). The smaller influx is attributable, of course, to the delay in arrival of Ca^{45} to the more centrally located fibers during a soak period limited to *ca.* 4.5 minutes.

The increment in calcium uptake as a result of potassium depolarization averages 0.013 μM/gm. This figure is undoubtedly smaller than it would have been had Ca^{45} completely filled the extracellular space at the time of potassium application. The smaller passive influx in these experiments is not quite an index of the diffusion limitation in the potassium experiments, for potassium was applied after 2.5 minutes, whereas the influx figure is based on a total of 4.5 minutes. Consequently, under equilibrium conditions in the extracellular space, calcium uptake during potassium depolarization may be expected to be

comparable more to a threefold than to a twofold larger figure; *i.e.*, 0.04 μM/gm. This amount of calcium is approximately that estimated as bound more firmly to the fiber surface sites (34), which suggests that these sites are the source of the entering calcium. As will be shown shortly, a similar conclusion may be drawn for the increased calcium influx during the twitch.

DISCUSSION

Our findings are in keeping with the hypothesis that the entry of calcium during the impulse and depolarization initiates contraction and contracture. The increased entry of calcium in nitrate solution could also account for the greater twitch and the prolonged contractile active state. Thus, the prolonged active state, to which is attributed the greater twitches (25), could be a consequence of a greater "slug" of entering calcium that requires more time to be ejected or bound by intracellular binding sites (*cf.* reference 8). The larger entry of calcium in nitrate, which is more polarizable than chloride, was predicted on the basis of improved binding of calcium to the membrane and easier association of these ions to form ion pairs in the membrane during decline of membrane potential (32). Another possibility is that nitrate, by augmenting the negative after-potential (1), increases the degree of depolarization and hence accelerates the entry of calcium; in this case the greater depolarization might be acting either by maintaining a greater permeability to ions generally or by improving ion pair formation. However, an explanation in terms of a greater depolarization is not satisfactory. Lubin (21) has reported that the action of nitrate on the negative after-potential of sartorii of *R. pipiens* (in contrast to the reports based on European amphibians) is so small as to be uncertain. Moreover, potassium contracture, which as in heart (23) is dependent on extracellular calcium (7), is also enhanced by NO_3^- without appreciable alteration of the potassium depolarization (13, 27). Finally, nitrate *decreases* the conductivity of the membrane at rest (24), at least in part that contributed by chloride (10), and thereby may be responsible for the larger after-potential (32); whether under these circumstances "permeability" to the calcium ion is increased by depolarization remains to be demonstrated. Consequently, the concept of calcium entry as an ion pair with nitrate appears to be more consistent with available facts. Measurements of the entry of nitrate (or of other highly polarizable anions) during activity for comparison with the entry of chloride (4, 5) may also prove useful in resolving the events taking place.

The experiments tabulated in Table II show that while depolarization is a necessary condition for increased calcium entry, it is not sufficient. Thus, in the experiments in which Ca^{45} was applied simultaneously with K^+, there is not the slightest indication of increased calcium entry, although for the short soak periods there was ample time for passive uptake. On the other hand, when Ca^{45} was given a little time to enter the extracellular spaces, there was a large

increase in uptake. This is most easily interpreted as the consequence of Ca^{45} first exchanging with Ca^{40} bound in the surface of the muscle fibers, from which the Ca^{45} that enters during depolarization is derived. In the first series of Table II such exchange had not occurred at the time of depolarization, hence the increased entry of calcium was not reflected by the Ca^{45}. This series also shows that calcium influx during the later period of depolarization is not appreciably different from that before depolarization. One is compelled to conclude, therefore, that the accelerated entry of calcium during potassium depolarization occurs only at the beginning and is not sustained. Thus, the situation appears to be that the increased entry of calcium during potassium depolarization results from the release of calcium from the surface of the fibers, and its failure to be sustained is due to the depletion of this surface calcium. Whether competition by potassium for the calcium-binding sites is partly responsible for the depletion of the surface calcium remains to be determined; this is suggested by the maintained increase in Ca^{45} entry at lower (20 mM/liter) potassium concentration (Table I). In any case, the time course of the contracture appears attributable to the entry of calcium, which is subsequently removed by being bound or by increased outflux (32).

Methods which are now available for measuring the calcium bound to the fibers (34) can be employed to verify these conclusions. They have been successfully used to explain the relation between the calcium concentration of the medium and passive and activity influxes. Thus, the failure of the calcium influx of activity to increase with the calcium concentration of the medium is consistent with the release of the entering calcium from sites saturated with calcium at the surface of the fibers (32). That such superficial sites are indeed saturated by the normal calcium level of the medium has now been shown (34). The dependence of passive calcium entry on calcium concentration, on the other hand, demonstrates that this penetration involves different sites in the membrane.

The calcium content of muscle fibers has been reported to be unchanged by stimulation (5). This may well be the consequence of a cancellation of the influx by a subsequent rise in outflux. Woodward reported an increase in outflux (35), which we have confirmed (34). But, even if there were no increase in outflux the entry of calcium would be near the borderline of detectability. Thus, our influx figures per twitch are *ca.* 0.015 of those for net sodium entry (see pages 216 and 217 in reference 32); even if the sodium content of whole muscle were doubled by activity—representing an increment of 20 μM/gm. in cat muscle (4)—this would correspond to a calcium uptake of 0.3 μM, or about 30 per cent or less than that found in mammalian and amphibian muscle (5, 8, 9).

The significance of the increased release of Ca^{45} with activity is presently being studied and cannot be discussed in detail here. However, it must be pointed out that it may not actually represent an increase in the transfer *across* the membrane but rather merely an outward component complementary to the inward component of

Ca^{45} movement when Ca^{45} is released briefly from its binding sites during activity. On the other hand, if an increase in the outward transfer from intracellular sites does occur, it must take place after that of influx if the latter is to exert its full intracellular effect. A precedent for the temporal separation of influx and outflux of ions, albeit of different ion species, has been set by studies of the spike (32). Thus, calcium entry might be by way of the sodium channels and exit by way of the potassium channels during the rising and falling phases of the spike. Or the increased exit could be a consequence of a substantial increase in ionized calcium in a superficial layer (cortex) of the myoplasm resulting from the increased entry. The possibility of a spatial separation of the two fluxes must not be overlooked, especially in view of early microincineration studies (see below). In any case, the time course of increased outflux suggests a physical rather than a metabolically driven process (34). Further discussion of this must await publication of our outflux data.

The influxes in squid giant axon (14) are compared with those in muscle in Table III. While the resting influxes are about the same, activity leads to a

TABLE III

Comparison of Calcium Influxes in Squid Giant Axon and Frog Sartorius Muscle

	Resting	Stimulated
	μmole/cm.² sec.	*μmole/cm.² impulse*
Giant axon (14)	0.076	0.0062
Muscle	0.094	0.20

30-fold greater influx in muscle fibers. In view of the absence of a saturation effect on influx by the calcium of the medium during activity of squid axons (14), it is tempting to propose that the saturation of surface sites that bind calcium plays the important role in muscle of increasing the amount of calcium entering per impulse, and that this represents a specialization related to the initiation and perhaps regulation of contraction. Other differences between squid axons and muscle, for example the absence of an increased outflux with activity in the former (14), also suggest a calcium mechanism in muscle specialized for contraction. However, the generality of the findings on the giant axon for other nerve fibers, particularly those that are normally in a medium much lower in calcium (*e.g.* from vertebrates), remains to be determined before the differences can be ascribed definitely to specialization for contraction.

On qualitative and quantitative grounds, then, our findings are consistent with the view that calcium entry initiates and may exert control of contraction. Two additional questions of a quantitative nature may be posed: How can calcium, released from the surface, act rapidly enough to activate all the contractile protein of large fibers despite diffusion limitations (12, 22)? And how can the small amount indicated by the influx data, which for each twitch repre-

sents only a fraction of a per cent of the total exchangeable calcium (*cf.* reference 8), be effective?

Recent studies, especially those by Huxley and Taylor (17), indicate that the striations of vertebrate muscle are partly a specialization that permits the action of depolarization at the surface to be transmitted quickly towards the center of the fiber. Thus, a fine electrode placed at different points along the sarcomere reveals that surface depolarization is effective only over a Z line and affects only the I band within which the Z line (actually a disc) is centered. Only patches of varying length along the circumference of the Z line or disc respond to depolarization. The suggestion has been made that "tubules" seen within the Z line (26) transmit the surface action. Only the I band (actually a cylinder) appears to undergo shortening (15, 16), for the A bands on either side are pulled symmetrically to the Z line in the center of the I band.

The most obvious proposal to be made, then, is that the active patches over the Z line correspond to the sites saturated with the calcium liberated into the fiber during excitation or during potassium contracture. The calcium released into the Z line cannot be expected to diffuse very far radially. However, this release into the Z line may cause a chain-like reaction whereby calcium, normally bound in a metastable form in the "tubules," is displaced from its sites in a wave traveling centrally from the periphery; such freed calcium could then diffuse longitudinally along the I band to actuate contraction. Diffusion distances would thus be no greater than half a sarcomere, or about 1 micron. More complicated situations involving the release of other substances from the Z line may certainly be visualized, but these are not justified as yet, especially in view of the following considerations concerning calcium distribution and the sensitivity of contractile protein to calcium entry.

Calcium was early shown to be the only physiological ion that causes shortening of muscle fibers when injected at low concentrations (11). This is consistent with recent evidence that protoplasmic calcium is largely in bound form (3, 9, 14). If, in addition, the calcium is lacking from regions in which sensitivity to calcium is required, *e.g.* the I band, then there appears to be almost no limit to the calcium sensitivity that may exist. It is interesting that the careful microincineration studies of muscle over 25 years ago (28), later confirmed (2), showed a distribution of multivalent ions consistent with these requirements. Thus, an ash attributable to calcium and/or magnesium was found in the A bands but not in the I bands. Moreover, the ash was also seen as fine lines corresponding to the Z lines and to the surface of the fibers. Of course, more recent histochemical methods that will avoid the possibility of calcium loss or translocation and that will distinguish between calcium and magnesium should be applied, especially to muscles in a state of contracture as well as at rest. Nevertheless, the early findings may be tentatively taken as support for the views developed here.

Ionophoretic injection of calcium from microelectrodes (22) suggests that 0.05 $\mu\mu$M/sec. is the minimal rate needed to induce localized contraction in frog fibers. But this must be regarded as an upper rather than a lower limit of sensitivity to calcium, for the injection was apparently not restricted to the I band, which may be more sensitive, nor was the possibility examined that the presence of the pipette reduced the sensitivity to stimulation *via* the Z line. In any case, it is of interest that, with the most favorable calculation, *i.e.* assuming calcium enters only during the spike (in 1 msec.) and diffuses only into the I band (one-third of a sarcomere), the rate of calcium entry per sarcomere calculates to 0.004 $\mu\mu$M/sec., or about a tenth the injection figure. The discrepancy is even greater if allowance is made for the injected calcium acting in a more limited region than a sarcomere (22). But a more careful examination of the minimal amounts of injected calcium required for shortening appears desirable before this difference can be considered a serious objection.

It is of interest that A. Huxley's observations of the shortening of the I band with the interference microscope suggest the loss of water, possibly with cellular constituents, during contraction, for the refractive index of the I band is found to be reduced briefly upon subsequent relaxation of the band. The increased outflux of calcium we have observed during isotonic shortening may be associated with such losses.

In conclusion, the findings to date support predictions that an increased entry of calcium underlies the development of the contractile response. It may be pointed out, too, that more detailed theoretical considerations, such as those that attribute the increased calcium entry to ion pair formation with anions due to collapse of the steep electrical gradient across the fiber membrane (28) provide a basis for the greater entry of calcium in nitrate solutions during contraction, described above, and for the smaller depolarizations that suffice in these solutions to produce potassium contracture (13). In these cases, nitrate is a more polarizable anion and therefore forms complexes and ion pairs more readily upon removal of the dissociation effect of the membrane potential, which across a membrane of 100 A is equivalent to a field of 100,000 volts/cm. (32). The possibility cannot be ruled out that calcium is accompanied by another membrane component that more specifically activates contractile protein. But the simpler view remains preferable until additional studies can show that calcium movement is not both necessary and sufficient to link membrane events with contraction and contracture.

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