Calcium Influx in Contracting and Paralyzed Frog Twitch Muscle Fibers

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ABSTRACT Calcium uptake produced by a potassium contracture in isolated frog twitch fibers was 6.7 ± 0.8 pmol in 0.7 cm of fiber (mean ± SEM, 21 observations) in the presence of 30 μM D600. When potassium was applied to fibers paralyzed by the combination of 30 μM D600, cold, and a prior contracture, the calcium uptake fell to 3.0 ± 0.7 pmol (11): the fibers were soaked in ^{45}Ca in sodium Ringer for 3 min before ^{45}Ca, in a potassium solution, was added for 2 min; each estimate of uptake was corrected for 5 min of resting influx, measured from the same fiber (average = 2.3 ± 0.3 pmol). The calcium influx into paralyzed fibers is unrelated to contraction. This voltage-sensitive, slowly inactivating influx, which can be blocked by 4 mM nickel, has properties similar to the calcium current described by several laboratories. The paired difference in calcium uptake between contracting and paralyzed fibers, 2.9 ± 0.8 pmol (16), is a component of influx related to contraction. Its size varies with contracture size and it occurs *after* tension production: ^{45}Ca applied immediately after contracture is taken up in essentially the same amounts as ^{45}Ca added before contraction. This delayed uptake is probably a "reflux" refilling a binding site on the cytoplasmic side of the T membrane, which had been emptied during the prior contracture, perhaps to initiate it. We detect no component of calcium uptake related to excitation-contraction coupling occurring before or during a contracture.

INTRODUCTION

The intracellular functions of most cells are under some form of extracellular control mediated by the cell membrane, often ultimately under the control of the nervous system. A. V. Hill (1948, 1949), working on skeletal muscle, was perhaps the first to understand the physical need for a specialized biological mechanism coupling the cell's membrane to its intracellular organelles (compare Heilbrunn and Wiercinski, 1947). Although the role of extracellular calcium in the coupling mechanism of skeletal muscle has been studied intensively for nearly 30 years (see Sandow, 1965, and reviews in Peachey and Adrian, 1983), the multiplicity of calcium actions irrelevant to coupling has obscured the underlying mechanisms. The role of calcium in the coupling mechanisms of other tissues

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(e.g., Rasmussen, 1981; Rubin, 1982; Cheung, 1982; Bianchi and Frank, 1982; Fleckenstein, 1983; Baker and Knight, 1984; Rink and Hallam, 1984) seems simple compared with its role in muscle, perhaps because possible irrelevant actions of calcium have not yet been investigated.

The transverse tubular (T) system, an invagination of the surface membrane (Peachey and Franzini-Armstrong, 1983) of striated muscle, is separated from the calcium storage and release organelle (the sarcoplasmic reticulum [SR]; Martonosi and Beeler, 1983) by a morphological and conceptual gap (Peachey and Adrian, 1983, p. 275–486). Depolarization of the resting potential across the transverse tubular membrane is followed by nonlinear movement of charge bound within the T membrane (Schneider and Chandler, 1973); subsequently, calcium is released from the SR. The mechanism linking T membrane voltage to SR calcium release is the first messenger in this system. The calcium released by the SR is a second messenger, which converts a membrane event into a chemical signal recognized by the contractile apparatus. Calcium released from the SR binds to troponin on the thin filaments of the sarcomere, producing a long-range conformational change at the active site of the thin filament, which leads to cross-bridge binding, ATP splitting, tension production, and shortening.

Several authors (e.g., see reviews in Bianchi and Frank, 1982) have suggested that calcium influx ("trigger calcium") is the messenger between T depolarization and calcium release from the SR; indeed, calcium has long been known to enter the muscle cell during contraction (Bianchi and Shanes, 1959), and calcium itself can induce a massive release of messenger calcium from the SR (reviewed in Stephenson, 1981; Fabiato, 1983). But the naive view that calcium entry across the outer or T membrane immediately releases calcium from the SR is not easy to support: frog skeletal muscle contracts in calcium-free solutions (Curtis, 1963; Armstrong et al., 1972; Lüttgau and Spiecker, 1979; Miledi et al., 1984), and the large calcium currents found in skeletal muscle evidently have no role in excitation-contraction coupling (e.g., Almers et al., 1981; Gonzalez-Serratos et al., 1982; reviewed in Stefani and Chiarandini, 1982).

Schneider and Chandler (1973; see review of Schneider, 1981) suggested that calcium release from the SR membrane was under the direct control of nonlinear charge movement in the distant (20 nm) T membrane, invoking the translation of a 30-nm "rigid rod" as the molecular messenger of this remote control. Unfortunately, no structural or molecular candidate for the rigid rod has been found (Somlyo, 1979; Eisenberg and Eisenberg, 1982; Mitchell et al., 1983; Franzini-Armstrong and Nunzi, 1984), and the mechanisms and messengers coupling charge movement in the T membrane and calcium release from the SR remain to be discovered in skeletal muscle.

A variety of experiments (reviewed in Schneider, 1981) support the view that charge movement is intimately related to calcium release from the SR. For example, treatment with D600 (methoxyverapamil, a tertiary amine) has been found to block charge movement under the same peculiar conditions in which it reversibly blocks excitation-contraction coupling (Eisenberg et al., 1983; Hui et al., 1984). Since D600 is a calcium entry blocker in several tissues (Fleckenstein, 1983; Schwartz and Taira, 1983), it seemed worthwhile to compare its effects

on calcium movement and contraction in skeletal muscle. In particular, the reversible paralysis produced by D600 allows us to distinguish the calcium influx related to excitation-contraction coupling from that unrelated to coupling.

Our results show three components of calcium influx: one, related to excitation-contraction coupling, occurring *after* a contracture; a second, produced by depolarization, but unrelated to coupling; and a third, the resting influx. We can detect no (so-called "exchange") influx produced directly by the rise of sarcoplasmic calcium. Nor can we detect any trigger influx, i.e., we observe no influx related to coupling occurring before or during contracture. Perhaps the delayed flux refills an internal store that is depleted when the T membrane is depolarized, charge movement occurs, and the muscle produces a prolonged contraction.

METHODS

Single twitch muscle fibers were skillfully dissected from the tibialis anterior muscle of *Rana temporaria* by Mr. R. McCarthy. All results reported are from fibers that contracted at the end of the experiment.

Solutions

All solutions contained D600 (usually 30 μM), a gift of Prof. Dr. R. Kretzchmar, Knoll AG (Ludwigshafen, Federal Republic of Germany). The frog Ringer contained 120 mM Na, 2.5 mM K, 1.8 mM Ca, 121 mM Cl, and 3 mM phosphate buffer at pH 7.2. Two radioactive solutions, each containing 10 mCi ^{45}Ca in ~1 ml final volume, were prepared using the purchased radioisotope as the sole source of calcium. We typically purchased the isotope as 0.1 ml of 5 mg/ml solution of calcium chloride containing radioactive ^{45}Ca of specific activity 20 mCi/mg, the rest of the calcium being nonradioactive carrier ^{40}Ca. One solution, called ^{45}Ca/Na, was a radioactive Ringer described above; the other solution, called ^{45}Ca/K, contained 240 mM potassium methanesulfonate. Although concern has been expressed in the muscle literature (Graf and Schatzmann, 1984) about the effect of methanesulfonate anion on the activity of calcium ion, workers in other fields (Dani et al., 1983) have shown this effect to be negligible.

Apparatus

The experimental apparatus shown in Fig. 1 is similar to that used by Curtis (1966), Hodgkin and Horowicz (1959), and Keynes (1951). The Geiger-Müller (GM) tube (model 704, The Nucleus, Oak Ridge, TN) has an outside diameter of 0.86 cm, giving an effective counting dimension of 0.7 cm along the length of muscle fiber, with a background counting rate of 1–2 cpm. The dissected muscle fiber rested without damage on Parafilm, which covered the GM tube and the bottom of the chamber. The vertical position of the fiber was measured in an eyepiece graticule of a binocular microscope gazing through the glass front of the chamber. Maintaining a constant distance between the fiber and the counting window from one influx run to the next was important because half the beta particles produced by the disintegration of ^{45}Ca are absorbed in just 47 μm of Ringer solution. The GM tube was tipped forward at ~15° so that a fiber resting on Parafilm positioned at a given vertical location was also positioned uniquely along the front-to-back axis.

Ringer solution flowed across the fiber and the face of the GM tube at a rate of 1–2 ml/min, which was sufficient to clear the chamber of radioactivity in 1–2 min; in rare disasters, a fiber was damaged and the counting rate fell to background in <5 min. Counting rates from healthy fibers fell much more slowly (see Figs. 2–7). The fiber was

mounted by aluminum foil clips around the tendons, which were attached to a strain gauge and a hook, which in turn were attached to a micromanipulator. The fiber was stretched to 1.5 slack length and positioned front to back over the GM tube so that at the very least 20 μm of fiber extended beyond the outer edge of the GM tube. For this reason, our measurements are not sensitive to ^{45}Ca bound to the tendons. In cases where the fiber was shorter than the diameter of the tube, the fiber was moved forward off the center of the tube face and the counts measured were multiplied by a factor equal to (effective tube diameter = 0.7 cm) ÷ (length of fiber over window of the tube).

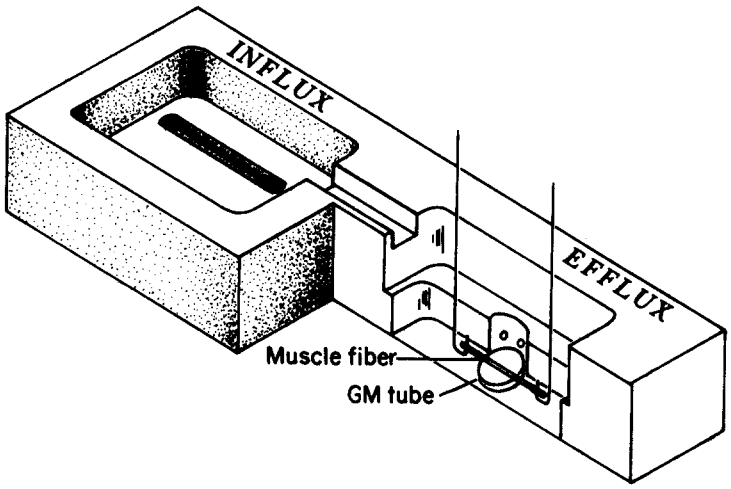

FIGURE 1. The apparatus. The influx cell (stippled) on the left contains a Teflon floor with a V groove, two platinum electrodes for stimulation, and a heating element to revive paralyzed fibers. The efflux cell on the right has a vertical glass front (not shown) so that the position of the single muscle fiber above the sloping face of the GM tube can be viewed with a stereomicroscope. Thin Parafilm covers the GM tube and the floor of the chamber. Ringer solution flows into the chamber through two holes behind the GM tube, and the fluid level is maintained by suction at the level of the step. The muscle is held between a force transducer and a fixed hook, both of which are attached to an overhead micromanipulator, which allows both individual adjustment and grouped movement to the influx chamber. Before transfer between chambers, the fluid level was raised and the fiber moved through the canal connecting the two chambers; it never was taken through an air-water interface.

The basic plan for all experiments is illustrated in Fig. 2. The muscle fiber was placed in the V groove of the influx chamber surrounded by a 0.5-ml pool of Ringer. Four drops (~0.5 ml) of ^{45}Ca/Na solution were added along the length of the fiber. To ensure mixing, three drops were then removed from one end of the pool and added to the other end. One 20-μl sample was taken from each end of the mixed pool; then the pool volume was reduced to 0.5 ml. The fiber was soaked in this solution for 3 min to allow isotopic equilibrium of the T system. (Nakajima and Bastian [1976] summarize the independent and consistent estimates from many laboratories, which show that monovalent ions diffuse in or out of the T system in ~15 s in a 140-μm-diam fiber. Eisenberg et al. [1977] review

structural models that predict such ion movements; Almers et al. [1981] discuss the diffusion of calcium.) After the 3 min, four drops of $^{45}Ca/K$ solution were added along the fiber, producing a contracture in an unparalyzed fiber. The asynchronous contracture resulting from this method of application of potassium was more variable than contractures produced by rapid changes of solutions with elevated potassium concentrations. Financial imperatives and concerns for safety forced us to conserve ^{45}Ca, precluding a rapid change of radioactive bathing solutions. Shortly after application of the $^{45}Ca/K$ solution, the influx pool was mixed and sampled. The measured dilution of ^{45}Ca from stock solution to influx pool allowed calculation of the potassium concentration around the fiber. The potassium concentration ranged from 73 to 210 mM, with a mean of 138. After the fiber had been in the $^{45}Ca/K$ solution for 2 min, the influx chamber was flooded with nonradioactive Ringer and the fiber was positioned over the GM tube. Counting began 10 min after the beginning of the influx period and continued for 50 min. The radioactivity of the fiber was estimated as the [(counts observed + counting interval of 10 min) − (background ≃ 1 cpm)]. These estimates were plotted at the midpoint of the counting interval. A straight line, fitted to the logarithm of these points by the method of least squares, was extrapolated back to the end of the influx period to estimate the radioactivity of the fiber at that time. The fiber illustrated in Fig. 2 took up 16.3 cpm during the previous influx period.

The sensitivity of the apparatus must be determined to translate the radioactivity (in counts per minute) observed with the GM tube into the amount of calcium uptake into the fiber (in picomoles). Fortunately, a single determination for each fiber was sufficient: we checked that our procedures accurately repositioned the fibers after each cycle of influx/efflux. Some fibers were repositioned over the GM tube without exposure to additional ^{45}Ca (as in Fig. 6*A*). The accuracy of repositioning was determined by extrapolating a straight line through the first set of counts forward to the times at which the counts were measured after repositioning. In 13 fibers—including 19 influx/efflux cycles with a total of 48 points—the average fractional difference between the extrapolated and measured values was 4.4 ± 3.9%, which is not significantly different from zero at $P < 0.05$.

The sensitivity of the apparatus shown was determined by comparing the counting rate of the central region of the fiber measured with the GM tube and the counting rate of the same length of fiber measured in the liquid scintillation system. At the end of the experiment (a time of 200 min in this experiment), when the fiber was counting 22.7 cpm over the GM tube, the center 0.7 cm of the fiber was placed in a liquid scintillation vial, solubilized, and counted (giving 10,610 cpm) in the same way as the diluted samples of influx solution. The sensitivity in a typical experiment (Fig. 2) was 2.24×10^{-3}, given by (final GM counts in the efflux period) ÷ (counts in the LS system). The specific activity of the influx solution is given by the total amount of calcium divided by the counting rate that would be observed with the GM tube and is estimated by the expression (moles Ca in sample of influx solution) ÷ [(counting rate of sample in the LS system) × (sensitivity)]. The uptake of calcium in moles is computed from the uptake measured in counts per minute (of the GM tube) using the specific activity. Corrections for efflux during the influx period were not significant.

Tension was measured during all experiments to determine the health and contractile state of the muscle fiber. For example, the peak tension measured during the first potassium depolarization in Fig. 2*A* (conditioning contracture) is 85 mg from a fiber with a diameter (measured at slack length) of 100 μm, which implies a diameter of 81 μm and a specific tension production of 1.6 kg/cm^2 at the 1.5× stretch we routinely used. (The calculation was made assuming constant volume with stretch [see Elliott, 1973; Matsubara

and Elliott, 1972].) The average peak contracture tension for the conditioning contractions was 2.3 ± 0.2 kg/cm^2, which is lower than the figure of 3.5 reported by Caputo (1972), and Hodgkin and Horowicz (1960), almost certainly because of the reduced number of actin-myosin cross-links able to generate tension in our stretched fibers with reduced overlap of thick and thin filaments (Gordon et al., 1966). Unfortunately, the method of application of potassium precluded detailed analysis of the contracture waveform and sometimes may have decreased the peak tension recorded.

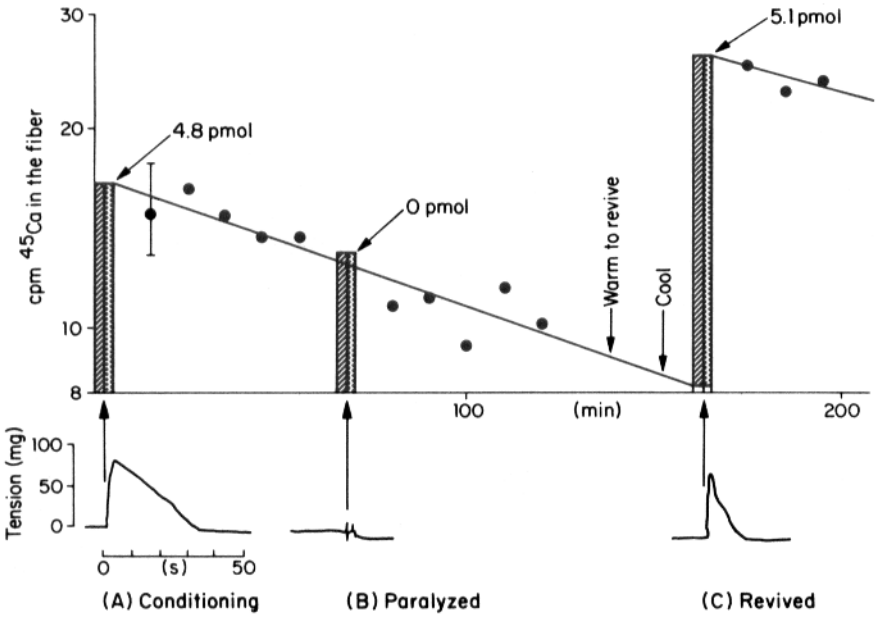

FIGURE 2. Paralysis abolishes potassium-stimulated calcium uptake. ^{45}Ca was added in Na Ringer (cross-hatched bars) for 3 min before 240 mM K (in ^{45}Ca) was added (stippled bar) to depolarize the fiber. In the first influx (*A*), the fiber contracted and took up 4.8 pmol Ca. In the second influx cycle (*B*), no tension was generated because the fiber was paralyzed by the combination of D600 (50 μM), cold, and the prior contracture. In this particular paralyzed fiber, no additional calcium entered the fiber in the second influx cycle. The fiber was warmed to revive contractile ability and then recooled. It took up 5.1 pmol Ca in the indicated contracture. All measurements were at 3°C and the standard error of counting of the first point is shown by the vertical bar. Fiber diameter, 100 μm.

Estimates of experimental parameters are given as means ± SEM where SEM is the standard error of the mean, that is, the estimate of the standard deviation of a single observation divided by the square root of the number of observations. Statistical comparisons of means were done by the Student's two-tailed *t* test.

RESULTS

^{45}Ca Influx and D600 Paralysis

In frog skeletal muscle, reversible paralysis is caused by the combination of 5–50 μM D600, cold (5°C), and a preceding (paralyzing or conditioning) contrac-

ture (Eisenberg et al., 1983). This combination interferes with the excitation-contraction coupling sequence at one of its earliest stages, the stage coupling T membrane potential to the nonlinear displacement of charge within the T membrane (Hui et al., 1984). Displacement of charge bound within a membrane protein (i.e., capacitive current) is a likely cause (and effect) of a conformational change driven by depolarization of the T membrane potential (Armstrong, 1977). Both charge movement and contraction revive after the fiber is warmed (to ~15°C for at least 10 min), even in the presence of D600. Fig. 2 shows the experimental protocol of drug application, conditioning contracture, paralysis, and revival by warming; it also shows the uptake of ^{45}Ca produced by potassium depolarization in each condition. Please note the logarithmic scale and location of baseline when worrying about the scatter in the points.

During the first measurement of influx (Fig. 2*A*), the fiber was bathed in frog Ringer containing radioactive calcium (^{45}Ca/Na) for 3 min before a potassium-rich, radioactive calcium solution (^{45}Ca in 240 mM K called ^{45}Ca/K) was added to the small pool in the V groove to depolarize the fiber and initiate contracture. The ^{45}Ca/K was applied for 2 min. The fiber took up 4.8 pmol calcium during the 5-min period the fiber was in ^{45}Ca: during 3 min of that period, it was at rest; during ~30 s of that period, it contracted; and ~1.5 min of the period was after the contracture.

After this one contracture, the fiber was paralyzed because of the D600, the cold, and the preceding contracture; it did not contract when ^{45}Ca/K was applied again (Fig. 2*B*: after a presoak in ^{45}Ca/Na). The uptake of ^{45}Ca in this "paralyzed contracture," as we call it, was zero (Fig. 2*B*) in this particular fiber. At this time, the fiber was warmed to revive contractility. It was then recooled so a comparable third calcium uptake measurement could be performed (Fig. 2*C*). The calcium uptake in the revived contracture was now 5.1 pmol, essentially the same as the calcium uptake during the conditioning contracture, although the contracture in this particular experiment was shortened compared with the original, probably because of a warmer temperature (Caputo, 1972), not fiber deterioration. The warmer temperature was due, no doubt, to the impatience of the junior author to get on with the experiment! Most of the fibers showed little sign of deterioration during the experiment.

In two fibers (Table I: 10-7, 123 μm and 10-18, 152 μm), α-bungarotoxin was added to block acetylcholine channels (Changeux et al., 1970), and tetrodotoxin to block fast Na channels (Narahashi et al., 1964). Other fibers were studied in the presence of the calcium entry blocker diltiazem at 3 μM. Although these drugs block channels that are possible routes of significant calcium uptake, particularly in the paralyzed fiber, our data show no effect on calcium uptake in contracting or paralyzed fibers.

Table I shows the fibers in which calcium influx was measured in three states: paralyzed, resting, and contracting, the latter phase including both conditioning and revived contractures because they were indistinguishable (ratio = 1.22 ± 0.14 in 24 observations). The table shows paired data where the influx measurements were made on the same fiber as well as pooled data from these and other fibers. In order to make the table easier to use, the uptake data have been

corrected for resting flux and normalized with respect to time in $^{45}Ca/K$, assuming that the resting flux occurred at a constant rate, independent of time. For example, the influx reported for contracting fibers was corrected from experiments like those in Fig. 3 by the formula {[uptake in pmol Ca (in 3 min $^{45}Ca/Na$, then 2 min $^{45}Ca/K$)] − [resting uptake in pmol Ca (in 5 min $^{45}Ca/Na$)]} ÷ (2 min). The calcium influx in contracting fibers was 2.4 ± 0.4 pmol Ca/

TABLE I

Calcium Influx (per Fiber)

Date	Diameter	Resting influx	Contracture influx	Paralyzed influx	Difference (contracture − paralyzed)
	μm	*pmol/min*	*pmol/min*	*pmol/min*	*pmol/min*
			Paired Results		
4-22	107	0.65	2.6	1.0	1.6
5-2	93	0.40	6.0	0.2	5.8
5-5	130	0.98	3.1	3.0	0.1
5-5	143	0.23	2.0	2.2	−0.2
5-18	131	0.23	3.2	2.8	0.4
5-18	159	0.22	6.6	2.3	4.3
5-19	130	0.12	0.7	0.6	0.1
10-6	162	0.07	3.5	3.0	0.5
10-7	190	0.57	1.5	−0.5	2.0
10-7	123	0.22	2.4	1.1	1.3
10-18	152	0.44	2.5	1.0	1.5
Mean	138	0.38	3.1	1.5	1.6
SEM	8	0.08	0.5	0.4	0.6
			Pooled Results		
Mean	140	0.46	3.3	1.5	1.5
SEM	5	0.05	0.5	0.3	0.4
n	34	18	21	11	16

Data are from a 0.7-cm length of fiber at 5°C.
"Contracture" data are from conditioning and revived contractures.
"Contracture" and "paralyzed" influxes have the resting flux subtracted as described in the text.

min (mean ± SEM; 21 values in 19 fibers). In paralyzed fibers, the uptake was 1.1 ± 0.3 pmol/min (16 in 16). A two-tailed Student's *t* test suggests that these means are significantly different with $P < 0.05$. The component of calcium uptake associated with contraction alone can be estimated by the difference between the calcium uptake in contracting and paralyzed fibers, as shown in the last column. The average of these paired differences is 1.5 ± 0.4 pmol/min Ca (16 measurements), which is significantly different from zero with $P < 0.005$.

We conclude that contracting fibers take up more calcium than paralyzed fibers: the excitation-contracture-recovery cycle is accompanied by an uptake of ~1.5 pmol/min more calcium than the depolarization cycle in paralyzed fibers.

This uptake, associated with excitation-contraction coupling, is only one component of the total uptake in a contracting fiber. Much of our experimentation is designed to identify and measure these components.

Resting Calcium Influx

Fig. 3 compares calcium influx in a conditioning contracture, in a paralyzed fiber, and in a resting fiber. After the conditioning contracture and the associated influx, here 14 pmol, the fiber was placed in ^{45}Ca/Na for 15 min and a resting

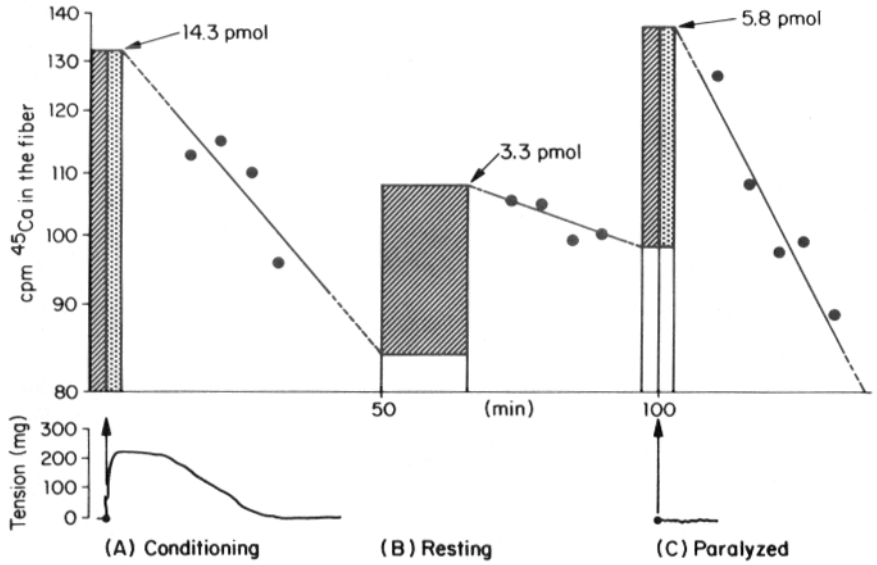

FIGURE 3. The Ca influx of the depolarized, paralyzed fiber is greater than resting. The protocol for *A* and *C* is the same as in Fig. 2. The difference represents a voltage-sensitive component of Ca influx unrelated to contraction. Resting Ca influx (*B*) was measured by applying ^{45}Ca/Na for 15 min. Temperature, 3°C; fiber diameter, 159 μm.

influx of 3.3 pmol was measured. The calcium uptakes shown in Fig. 3 during the conditioning and paralyzed contractures are among the larger values observed; nonetheless, the paralyzed fiber took up less than the contracting fiber. The resting calcium influx was measured in 18 fibers (Table I) and the average was 0.46 ± 0.05 pmol/min, at ~6°C. The average time constant for efflux, measured after a period of resting influx, was 75 ± 11 min in 8 measurements in fibers warmer than 6°C compared with a time constant of 152 ± 21 min in 14 measurements from fibers colder than 6°C.

Calcium entry into paralyzed fibers is significantly greater than the resting calcium influx. The difference, 1.1 pmol/min (labeled "Paralyzed influx" in Table I), is a component of calcium influx related to depolarization—not to contraction or excitation-contraction coupling, neither of which occurs in paralyzed fibers. We have demonstrated three components of calcium influx in a

potassium contracture: a resting component, a voltage-sensitive component unrelated to excitation-contraction coupling, and a component related to excitation-contraction coupling.

^{45}Ca/K Alone

^{45}Ca was applied as two solutions in the influx measurements reported so far: first, a ^{45}Ca/Na Ringer was applied for 3 min to establish isotopic equilibrium in the T system and then ^{45}Ca/K was added to depolarize the membrane potential.

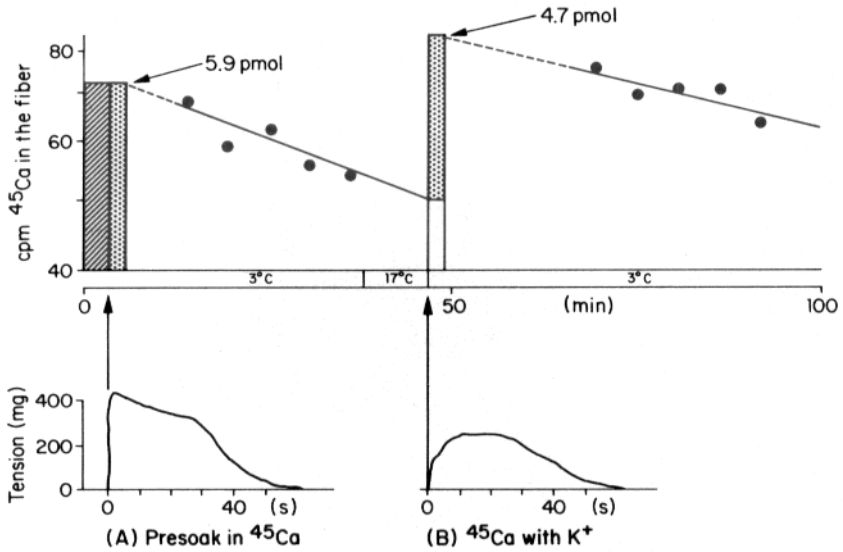

FIGURE 4. 3-min presoak in ^{45}Ca/Na slightly increases uptake during a potassium contracture at 3°C. In influx (*A*), ^{45}Ca was added first in Na Ringer (cross-hatched bar) for 3 min before 240 mM K was added (stippled bar) to the influx pool. In the second influx (*B*), ^{45}Ca was added only as ^{45}Ca/K; that is to say, isotope was present only in 240 mM K solution. The difference in tension records reflects the difficulties in our method of applying potassium. The similarity of uptake suggests that only a small fraction of the ^{45}Ca flux is entering the fiber through a rapidly and completely inactivating channel or during the rising phase of the contracture. Fiber diameter, 145 μm.

The effect of equilibration on calcium uptake was determined by comparing uptakes with and without an equilibration period. Fig. 4 compares an uptake with presoak (as described previously) to an uptake without a presoak, in which radioactive calcium was applied only as ^{45}Ca/K (Fig. 4*B*). The uptake of ^{45}Ca was slightly increased when a presoak in ^{45}Ca/Na preceded application of ^{45}Ca/K. The ratio of paired data was 1.1 ± 0.1 in four fibers, no different from 1 at $P < 0.05$. Contracting fibers (in both conditioning and revived contractures) took up 2.1 ± 0.2 pmol Ca/min when ^{45}Ca was added without a presoak (21 observations not corrected for resting influx). Paralyzed fibers took up 1.4 ± 0.4 pmol Ca/min (nine observations) with the same protocol. The comparable resting influx

is 0.5 ± 0.1 pmol Ca/min (six observations). The difference in the average values of calcium uptake in 21 contracting and 9 paralyzed fibers (without presoak) is 0.7 ± 0.4, which is somewhat less than the difference value in Table I, namely 1.5 ± 0.4 pmol Ca/min. The corresponding paralyzed influx (corrected for resting influx) is 1.0 ± 0.2 pmol Ca/min compared with 1.1 ± 0.3 for the fibers in Table I.

The rate of efflux of ^{45}Ca at 6°C apparently depended on the way ^{45}Ca was applied to the fiber. When ^{45}Ca was first applied as ^{45}Ca/K, the time constant for the subsequent ^{45}Ca efflux averaged 58 ± 13 min (16 measurements). When ^{45}Ca was first applied as ^{45}Ca/Na, the efflux was much slower, with a time constant of 152 ± 21 min (14 measurements). (Remember that in both cases the fiber is bathed in the same D600 Ringer when efflux is measured.) One interpretation of these results (which cannot be proven with the data at hand) is that ^{45}Ca is entering different "compartments" (as described by Curtis, 1970) when it is applied under different conditions. ^{45}Ca enters only the compartment with faster efflux when the isotope first confronts a depolarized fiber (when it is added only as ^{45}Ca/K). The isotope presumably enters both the slow and fast compartments when the isotope is applied first to a resting fiber (in the experiments with a presoak in ^{45}Ca/Na before application of ^{45}Ca/K), but the two time constants of efflux are not evident because of the inherent scatter caused by the low counting rate.

Partial Contractures

A few experiments, done early in the series, show a correlation of tension and calcium influx. In these fibers, D600, low temperature, and a conditioning contracture resulted in a reduction but not an abolition of tension. We are not sure why this occurred, but we suspect that the potassium concentration during the contracture was too low, particularly in view of the relatively warm temperature (Cooper et al., 1984). The tension records of two such experiments are shown in Fig. 5. Uptakes are given in counts per minute because an accurate specific activity was not obtained. In the two fibers shown, the uptake of ^{45}Ca was reduced to 48% of its value in the full contracture, while the peak tension was reduced by about the same amount. We conclude that reduced tension is accompanied by reduced ^{45}Ca entry. Note that the calcium uptake in the paralyzed state is much less here than in the average results shown in Table I. This was a consistent finding in our first set of experiments, done with our first sample of D600.

Nickel

The calcium influx into paralyzed fibers, 1.1 ± 0.3 pmol/min, flows through a system unrelated to contraction, probably activated by depolarization. Flux of this sort would flow through calcium channels unrelated to contraction, not blocked by D600, particularly if the channels did not inactivate during maintained depolarization. Channels of this sort are present in the T system of frog skeletal muscle (Almers et al., 1981; reviewed in Stefani and Chiarandini, 1982;

see also Donaldson and Beam, 1983) and nickel is known to block them without decreasing contracture tension (Caputo, 1981; Almers et al., 1981).

We added 4 mM Ni to our D600 (30 μM) Ringer, hoping thereby to reduce the potassium-sensitive calcium influx, unrelated to contracture, in both conditioning and paralyzed fibers. When D600 and nickel were added simultaneously, paralysis occurred but only after several contractures. If D600 was added after nickel, paralysis was not produced. Nickel, D600, and the muscle fiber evidently interact in a complex manner.

The fiber illustrated in Fig. 6 took up 0.5 pmol calcium when depolarized for 2 min in the paralyzed state and 7.5 pmol when depolarized in its revived state for the same time. (Resting fluxes were not measured from this fiber, nor were they measured in Ni/D600 solution, so data are given as total uptake in picomoles,

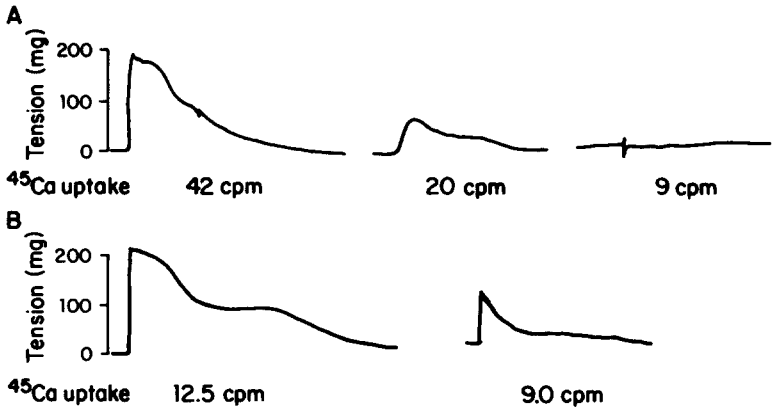

FIGURE 5. Ca uptake is reduced as tension and time-tension area declines in partial paralysis. Accurate specific activities were not obtained, so the uptakes are given as counts per minute in the fiber at the end of the influx period. Subsequent revival of the paralyzed fiber demonstrated the continuing good health of the preparation. Temperature, 5.5°C (*A*), 4°C (*B*); fiber diameter, 130 μm (*A*), 105 μm (*B*).

not as the rate of uptake in picomoles per minute.) Six fibers took up 2.1 ± 0.4 pmol (19 measurements) in 2 min when contracting in Ni/D600 solution, while four paralyzed fibers took up 1.4 ± 0.6 pmol (4 measurements) for the same time. The average resting calcium influx in 2 min was 0.9 ± 0.3 pmol.

The calcium influx in paralyzed fibers depolarized in a Ni/D600 solution is indistinguishable from the resting influx measured from other fibers. We conclude that nickel blocks ^{45}Ca uptake (irrelevant to contraction) just as it blocks calcium current (known to be irrelevant to contraction; Almers et al., 1981; Gonzalez-Serratos et al., 1982).

The voltage-sensitive component (irrelevant to contraction) can be estimated by the difference between the total calcium uptake in conditioning contractures in D600 (with isotope applied for 2 min as $^{45}Ca/K$), namely 2.1 pmol/min, and in contractures in Ni plus D600 (1.1 pmol/min), giving the estimate 1.0 pmol/min (35 measurements). This estimate is in good agreement with the other

independent estimate of the voltage-sensitive (contraction-unrelated) component of influx, namely 1.1 ± 0.3 pmol/min, determined from the difference between calcium influx in paralyzed and resting fibers.

Caffeine Contractures

A calcium influx associated with the excitation-contraction-recovery cycle has been demonstrated in Figs. 2–6. Caffeine releases calcium directly from the SR, bypassing the excitation step (Kovacs and Szucs, 1983, and references cited

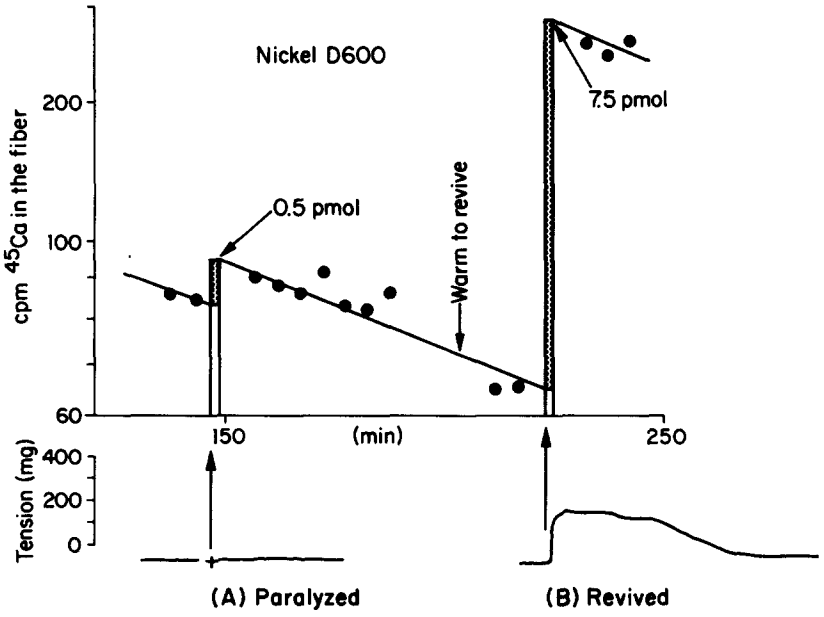

FIGURE 6. Ni reduced Ca influx into the paralyzed fiber to the level of resting influx. Ni increased the number of contractures needed before paralysis occurred—three were necessary in this fiber—and the associated Ca entry decreased progressively. After warming, the fiber was repositioned over the GM tube and the final two points of panel *A* were obtained. Clearly, the fiber can be accurately repositioned and the counts observed are reproducible (see Methods). The revived contracture (*B*) of the recooled fiber develops full tension and a large Ca influx. The temperature was 3.5°C, except during warming period. Fiber diameter, 155 μm.

therein). Thus, we hoped that measurements of calcium uptake induced by caffeine would separate fluxes that are the immediate result of the rise in sarcoplasmic calcium concentration (or the immediate result of mechanical activity) from those associated directly with excitation-contraction coupling. The frustration of this hope is described in the Discussion.

Calcium uptake was first measured in caffeine contractures in the presence of Na. After the fiber had been in ^{45}Ca/Na solution for 3 min, two drops of 15 mM caffeine solution were added to the approximately six drops of solution surrounding the fiber in the influx pool (see Fig. 1), giving a diluted concentration of

caffeine of ~4 mM. Caffeine contractures were terminated soon after 1 min by flushing with Ringer. Resting influxes were not measured, so the data are given as total uptake (in picomoles), not as the rate of uptake (in picomoles per minute). Five fibers took up 2.3 ± 1.1 pmol in caffeine/Na contractures in 2 min, compared with 8.5 ± 2.4 pmol Ca in 2 min when isotope was applied for 3 min as ^{45}Ca/Na and then for 2 min as ^{45}Ca/K, with a paired difference of 4.2 ± 1.4 pmol Ca in 1 min, which is quite different from zero at $P < 0.05$.

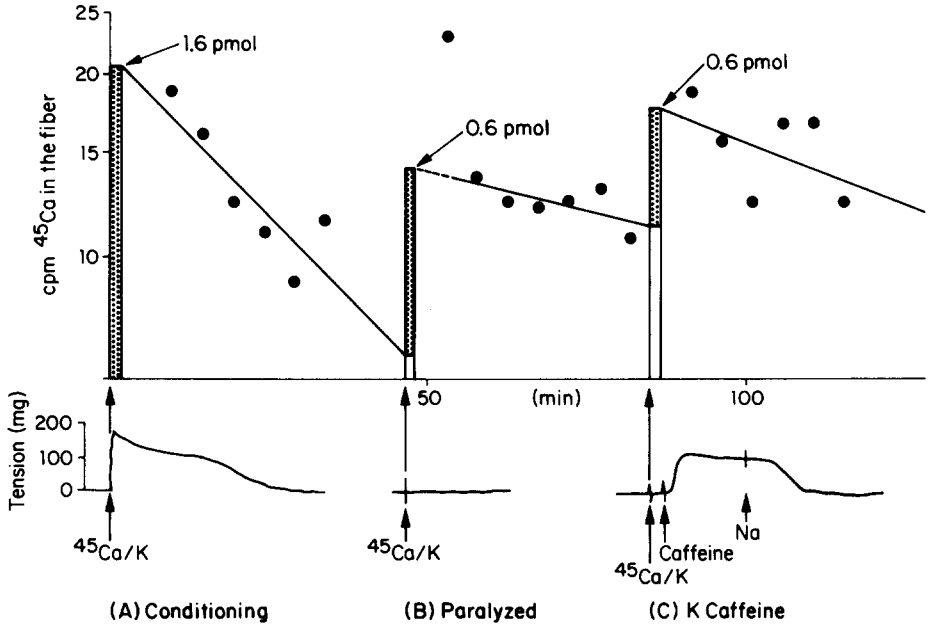

FIGURE 7. Calcium uptake in caffeine contracture (*C*) is no greater than calcium uptake produced by depolarizing a paralyzed fiber (*B*). Calcium uptake in the K/caffeine contracture of a paralyzed fiber is considerably less than calcium uptake in a potassium contracture (*A*). In *C*, the fiber was exposed to ^{45}Ca/K for 10 s before caffeine was added to the ^{45}Ca/K to produce the contracture. Both ^{45}Ca/K and caffeine were washed off by the Na Ringer solution to terminate the contracture. Temperature, 3.5°C; fiber diameter, 122 μm.

Calcium uptake produced by caffeine/K was compared with the calcium uptake produced by potassium in normal and paralyzed fibers (Fig. 7). In Fig. 7*C*, the paralyzed fiber was exposed to ^{45}Ca/K for 10 s before caffeine was added to produce a large contracture. The calcium influx produced by caffeine/K is no greater than the influx produced by potassium alone in the paralyzed fiber (which produced no tension: Fig. 7*B*); it is 1 pmol less than the influx produced by potassium alone, in the conditioning contracture (Fig. 7*A*). In six fibers, the calcium uptake in potassium contractures (with isotope applied only as ^{45}Ca/K) was 4.3 ± 0.7 pmol in 2 min, and in caffeine contractures (in paralyzed fibers in the presence of high potassium) it was 2.7 ± 1 pmol in 2 min. The paired

difference (for corresponding contraction time) is 1.5 ± 0.6 pmol Ca, which is much different from zero.

We conclude that the calcium influx associated with a potassium contracture is significantly larger than the calcium influx associated with caffeine contractures. We shall see, moreover, that the calcium influx produced by caffeine includes a component that is absent during a potassium contracture.

Timing of Calcium Uptake

The preceding experiments have identified a component of calcium influx related to either excitation or recovery. When potassium and ^{45}Ca are added together to a muscle fiber, depolarization of the T membrane occurs within milliseconds (reviewed in Nakajima and Bastian, 1976), while diffusion of ^{45}Ca into the T system takes 10–15 s (Almers et al., 1981). Tension rises within 20 ms, so the initial responses to depolarization, including the rising phase of tension, are completed before significant amounts of ^{45}Ca diffuse deep into the T system: rapidly inactivating channels in the T membrane will have opened and shut before the isotope arrives. Thus, the component of ^{45}Ca uptake correlated with excitation-contraction coupling is unlikely to flow through rapidly inactivating channels in the T membrane: this component of influx must flow during or after the contracture. We suspect in fact that a contraction-unrelated component of calcium influx (discussed later) flows through rapidly inactivating channels (Almers et al., 1981; review in Stefani and Chiarandini, 1982; see also Donaldson and Beam, 1983).

Fig. 8 investigates the timing of Ca uptake. The uptake shown in column *a* was measured by the protocol shown in Figs. 2*A*, 3*A*, and 4*A*. The isotope was present (in a sodium solution) for 3 min before the contracture and was present in a potassium solution for an additional 2 min, during which time the fiber contracted and relaxed. The data reported have been corrected for the resting uptake in the 3-min exposure to isotope before the contracture. The data shown in column *b* were measured from paralyzed fibers by the protocol shown in Figs. 2*B* and 3*C* and also corrected for the resting uptake, which occurred before the contracture. The data shown in column *c* were measured from conditioning contractures using the protocol shown in Figs. 4*B* and 7*A*. The data shown in column *d* were measured with the same protocol but from paralyzed fibers, as shown in Fig. 7*B*. Resting influx was not subtracted from the data in columns *c* and *d*. The data in columns *e* and *f* were measured by applying isotope (in a potassium solution) after a conditioning or paralyzed "contracture" (respectively), and allowing the fiber to remain in the isotope and potassium solution for 2 min. Resting influx was not subtracted. Consequently, the data shown in Fig. 7 have not been corrected for the 2 min of resting influx (0.9 pmol), which is likely to occur during the 2 min of potassium application.

The data in columns *a*, *c*, and *e* are directly comparable estimates of the influx in contracting fibers in different 2-min periods. Column *a* measures influx at the beginning of, during, and after the contracture; *c* measures influx during and after the contracture; *e* measures influx only after the contracture. The only time period these three columns share is the post-contracture period. Thus, we

conclude that most of the influx related to excitation-contraction coupling occurs after the contracture.

Calcium influx in paralyzed fibers (stippled bars, Fig. 8) is strongly dependent on the time period in which it is measured. It is greatest when the isotope is added before potassium (column *b*); it is reduced when isotope is added simulta-

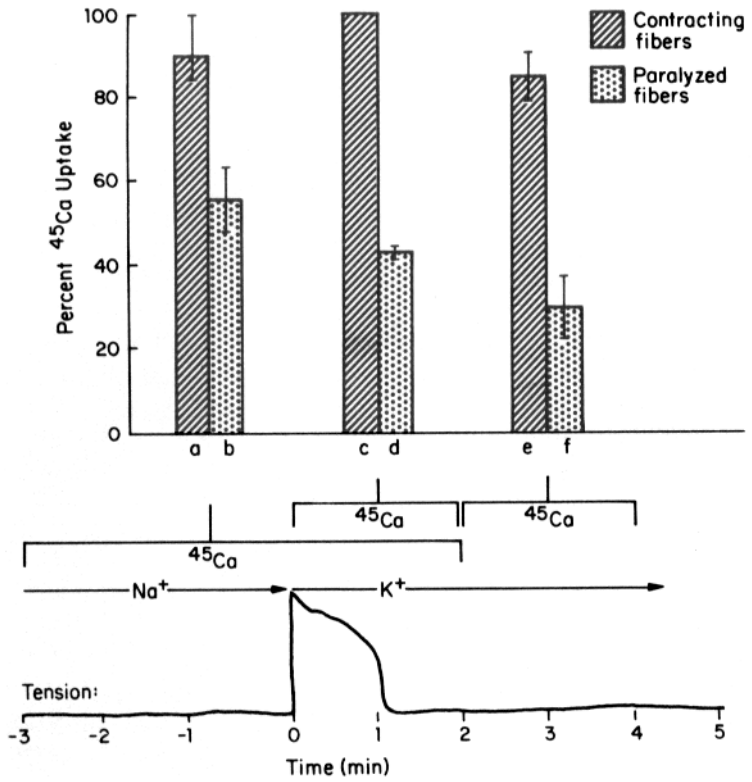

FIGURE 8. Timing of calcium uptake. ^{45}Ca was applied to fibers before, at the same time as, and after potassium depolarization. The precise protocols and correction for resting flux are described in the text. The calcium uptake in contracting fibers (hatched bars) was not significantly ($P < 0.01$) different in each of the three application periods: calcium is taken up if it is present during the post-contracture period. The contracture-related component of calcium influx is associated with recovery. The ^{45}Ca uptake in paralyzed fibers (stippled bars) is greatest when the isotope is added before potassium, and is significantly reduced when applied 1–4 min after depolarization. We believe this component of Ca influx, unrelated to contraction, enters the fiber through a voltage-sensitive channel that slowly but incompletely "inactivates" in the face of prolonged depolarization. Calcium influx in contracture *c* (produced by a 2-min soak in ^{45}Ca/K solution) was taken as the reference value (i.e., 100%). The data shown in columns *a* and *e* are from paired observations. The uptake in paralyzed fibers is given as a fraction of the contracting uptake.

neously with potassium (column *d*); it is further reduced when isotope is added after potassium (column *f*). The component of influx measured in paralyzed fibers is clearly not associated with excitation-contraction coupling, because neither coupling nor charge movement occurs in the paralyzed condition. This calcium uptake behaves as if it flowed through a voltage-sensitive channel that slowly inactivates with time.

We conclude that the calcium uptake associated with contracture (1.3 pmol/min) must occur after the contracture: the data show no sign of a component of calcium uptake contemporaneous and correlated with tension production. It seems that the flux from the extracellular space replenishes an internal calcium store depleted by the preceding contracture, a refilling influx.

Analysis of Calcium Influx

The calcium uptakes reported in the preceding experiments can be described as the composite of three parallel and apparently independent calcium influxes. In this section, we tease these three components apart quantitatively from measurements of total uptake under various conditions.

The first component, resting calcium influx, is classically presumed to flow at a constant rate as long as ^{45}Ca is present. The second component, calcium influx unrelated to excitation-contraction coupling, is presumably the result of channel activation by depolarization and is blocked by nickel. It decreases as these channels inactivate in the face of prolonged depolarization. The third component, refilling influx, is somehow related to excitation-contraction coupling, although it occurs after the contracture. We shall see that these three components of calcium influx account for all the calcium uptake observed under a variety of conditions.

Fig. 9 defines each protocol (*a–f*) used to measure calcium uptake in response to potassium. The components of calcium uptake in each protocol are shown above the corresponding tension tracing. The components might be different in size in different protocols, even though they are drawn similarly in the figure. The table below lists several estimates of each component of calcium influx. For example, the refilling component related to excitation-contraction coupling can be estimated in three different ways, using three independent combinations of the results of six distinct protocols. The three estimates shown are nearly equal. Similarly, the two independent estimates of the flux unrelated to coupling (made from independent combinations of the results from four distinct protocols) are indistinguishable. The similarity of these independent estimates supports our division of the total flux into three components and would be expected if the components represent flow through molecularly distinct membrane "channels" (i.e., transport systems).

The analysis can be checked in another way, by comparing the total uptake observed in a contracture (i.e., protocol *a*: shown on the last line of the table) with the predicted sum of its components, using only estimates that do not involve measurements with protocol *a*. The sum is shown on the second to last line. Note that measurements which do not involve protocol *a* are shown in italics in the table. The measured value of total uptake (2.1 ± 0.3 pmol/min) and

the independently predicted sum of the components (2.4 ± 0.9 pmol/min) are gratifyingly close and are not significantly different at $P < 0.01$.

Because this analysis accounts for all of the observed calcium uptake, we conclude that the calcium influxes produced by potassium under a variety of

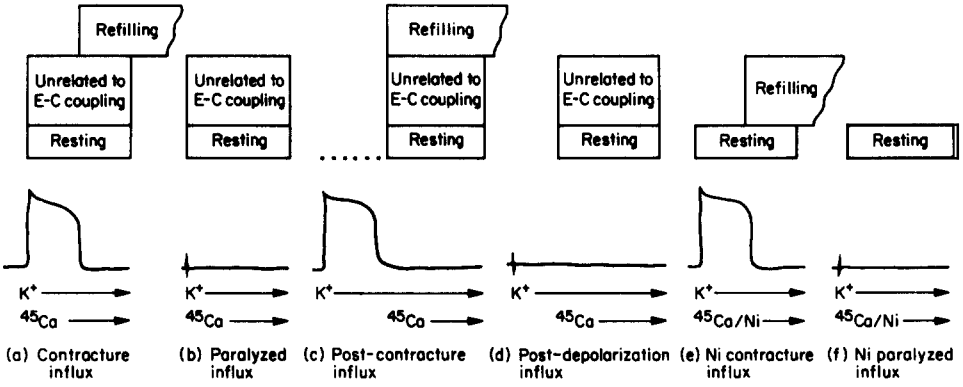

FIGURE 9.

Component	Measured by	Observations	Ca uptake
			pmol/fiber/min
Refilling	*c − d*	*15 averaged*	*0.7±0.1*
	e − f	*4 paired*	*1.0±0.4*
	a − b	**16 paired**	**1.3±0.4**
Unrelated to E-C coupling	*b − resting*	*11 paired*	*1.1±0.3*
	a − e	**35 averaged**	**1.1±0.3**
Resting	*^{45}Ca/Na*	*18 fibers*	*0.5±0.05*
Contracture influx	*Sum of components*	*30 fibers*	*2.4±0.85*
	Actual	**37 fibers**	**2.1±0.3**

Each protocol for the application of isotope is named below the tension record and the components of calcium influx are indicated above. Components might be different in size in different protocols, even though they are drawn similarly in the figure. A flat tension record indicates a paralyzed fiber. The timing of application of potassium and ^{45}Ca is shown below the tension line. Estimates of these three components are shown in the table along with the method of estimation. Italics are used to mark estimates that do not involve uptakes measured with protocol *a*. Agreement is good between the different independent estimates of refilling and coupling unrelated influx. The sum of the components of calcium influx (computed using only quantities in italics) is 2.4 pmol Ca/min, while the measured value of contracture influx *a* is 2.1 pmol/min Ca when corrected for resting flux. This latter value involves fibers exposed to both ^{45}Ca/Na and ^{45}Ca/K and fibers exposed just to ^{45}Ca/K. Each influx protocol is more precisely defined in one of the earlier figures and accompanying text: contracture (i.e., conditioning) influx is defined in Fig. 3*A*; resting influx, in Fig. 3*B*; paralyzed influx, in Fig. 3*C*; post-contracture influx, Fig. 8*e*; post-depolarization influx, Fig. 8*f*; Ni contracture influx, Fig. 6*B*; Ni paralyzed influx, Fig. 6*A*.

conditions and protocols are fully described by these mechanistically distinct components. Analysis shows no sign of an influx produced concurrently and directly by the rise of sarcoplasmic calcium, just as the experiments of Fig. 8 showed no sign of such a flux. The influx measured in caffeine contractures is discussed later.

In the previous paragraphs, we have assumed that the resting pathway for calcium influx continues during depolarization. The voltage-sensitive component, unrelated to excitation-contraction coupling, is assumed to flow through a separate "channel" and thus occurs in addition to the resting flux during depolarization. If the reader chooses to assume that the resting pathway disappears during depolarization, one estimate of the voltage-sensitive, contraction-unrelated component (namely b − *resting* in Fig. 9) becomes ~1.6 pmol/min. The other estimate (a − e in Fig. 9) is unchanged at 1.0 pmol/min. The contraction-related component remains between 1.5 and 1.1 pmol/min, depending on the data used.

DISCUSSION

Single frog twitch muscle fibers were depolarized by potassium in the presence of D600. Under these conditions, fibers contract once and then are paralyzed. This influx, related somehow to excitation-contraction coupling, occurs after relaxation and appears to be the refilling of an internal calcium store emptied in the previous contracture. We have demonstrated two other components: one, a calcium influx dependent on voltage but unrelated to excitation-contraction coupling; the other, a resting influx.

Components of Calcium Influx

The resting influx reported in this paper of 0.46 ± 0.05 pmol/min was measured at 3–6°C in the presence of 30 μM D600 and is suspiciously close to the value at 20°C reported previously (Curtis, 1966). These results suggest that neither temperature nor 30 μM D600 has a profound effect on resting calcium influx, thus supporting the classical treatment of resting influx (Hodgkin and Horowicz, 1959), which assumes that the resting influx continues unchanged during potassium depolarization. The resting influx is equivalent to a flux of 0.25 pmol/(s·cm^2 of outer surface area) or 0.03 pmol/(s·cm^2 of T and outer membrane), using the morphometric parameters of Mobley and Eisenberg (1975). In resting squid axon (Hodgkin and Keynes, 1957) and in frog ventricle (Niedegerke, 1963), calcium influx is somewhat greater, 0.1 pmol/s·cm^2.

Resting calcium efflux is strongly temperature dependent and clearly flows against a concentration and electrical gradient, which implies the presence of an active calcium transport system. A calcium-transporting protein has been reported in T membranes (the "Ca ATPase": Lau et al., 1977; Rosemblatt et al., 1981), but we speculate that another protein—the so-called "Ca/Mg ATPase" of the T membrane—although not yet shown to be a transporter, will eventually prove to be the energizer of active calcium efflux, either directly as a calcium pump or indirectly as a hydrogen ion pump creating a pH gradient, which in turn drives a Ca/H exchanger. Any vigorous calcium transport system in the T

system is likely to elevate the resting concentration of calcium in the T lumen above that in Ringer solution, diluting the specific activity of the ^{45}Ca and making our estimates of fluxes too low. Convection resulting from such active transport would have unpredictable effects.

Voltage-dependent Influx Unrelated to Excitation-Contraction Coupling

The voltage-sensitive component of calcium influx is unrelated to contraction and occurs during potassium application in both contracting and paralyzed fibers. We suspect that this flux flows through a subpopulation of calcium channels that do not fully inactivate upon depolarization even when treated with D600. Pharmacological measurements of nitrendipine binding sites (Fosset et al., 1983*a*, *b*) and electrical measurements of calcium current using voltage-clamp techniques (reviewed in Stefani and Chiarandini, 1982; see also Donaldson and Beam, 1983) show no shortage of putative calcium channels in the T membrane, even in the presence of D600 (Almers et al., 1981). Our best estimate of the voltage-sensitive uptake is 1.1 ± 0.3 pmol/min or 6.2 pmol/(min·cm^2 of T and surface membrane), determined from 11 paired values of influx in paralyzed and resting fibers (Table I) in the presence of D600. The corresponding mean calcium current of 10 nA/cm^2 of total membrane area is approximately three orders of magnitude less than the calcium currents observed with the voltage clamp near 20°C without D600. The calcium current declines precipitously as temperature is reduced (Cota et al., 1983) and is very small below 12°C (E. Stefani, personal communication).

While our methods are hardly able to determine the kinetics of this component of calcium influx, some conclusions can be reached. The ^{45}Ca uptake in paralyzed fibers (Fig. 8: stippled bars) is greatest when the isotope is added before potassium, and is significantly reduced when applied 1–4 min after depolarization. These channels evidently open quickly and inactivate slowly.

The function of this voltage-sensitive component of calcium influx is not clear, since it is unrelated to contracture (Almers et al., 1981; Gonzalez-Serratos et al., 1982; reviewed by Stefani and Chiarandini, 1982). One possibility is that the flux through this channel is needed to activate calcium-activated potassium channels, which are used to repolarize a fiber after depolarization (Fischman and Swan, 1967). Calcium-activated potassium channels are present in large numbers in the T membrane (Latorre et al., 1982; Magleby and Palotta, 1983). A mechanism of this sort might be involved in the late afterpotential following a train of action potentials (Kirsch et al., 1977, and earlier references cited therein); it might explain the reluctance of a fiber to repolarize after a potassium contracture in the absence of calcium ions or in the presence of some calcium channel blockers (Cooper et al., 1984). Nickel, a blocker of the calcium channel, slows repolarization after an action potential (Fischman and Swan, 1967).

Calcium Influx and Sarcoplasmic Calcium

Several colleagues have suggested that a component of the calcium influx we observed (say, in protocol *a* of Fig. 9) might be a direct result of the rise of calcium concentration in the sarcoplasm, perhaps the result of a membrane transport system that exchanged intra- and extracellular calcium. Different

protocols were designed to reveal and estimate such a component, called calcium exchange for short.

Experiments in which isotope is applied after contracture (Fig. 8) show that all the contraction-related uptake occurs after contracture, during recovery when the sarcoplasmic calcium is probably not much more than at rest and extra calcium exchange would not be expected. Analysis of calcium uptake (Fig. 9) into its components also shows no sign of calcium exchange: the uptake measured in a variety of conditions and analyzed in several different ways can be explained without invoking calcium exchange.

We now consider the calcium uptake measured in caffeine contractures, after remarking that no matter how it is interpreted, the calcium influx observed in a caffeine contracture is qualitatively consistent with the existence of a component somehow related to excitation-contraction coupling. Calcium uptake is smaller in either type of caffeine contracture than it is in potassium contractures. The difference in uptake in caffeine contractures with and without potassium probably reflects the direct effect of potassium depolarization on calcium uptake (seen also when potassium is applied without caffeine). Uptakes in either type of caffeine contracture are, however, quantitatively inconsistent with the estimates of components given in Fig. 9, which suggests that caffeine is having some direct effect on calcium movement across the sarcolemma, in addition to its well-known effect on the SR. Other work shows that caffeine binds to adenosine receptors in neural membranes (Snyder, 1984, and references cited therein) and affects calcium currents across the plasma membranes in a number of cells (neurons: Henon and McAffee, 1983; cardiac muscle: Blinks et al., 1972; smooth muscle: Burnstock, 1972, 1976; skeletal muscle: E. Stefani, personal communication). We performed two experiments showing a roughly threefold increase in calcium influx in the presence of 2-chloroadenosine, a drug known to bind to adenosine receptors and to affect calcium currents in neural preparations. Thus, the extra calcium uptake measured in caffeine contractures is probably the result of a direct action of caffeine on methylxanthine (e.g., adenosine) receptors on the sarcolemma, an action not present in potassium contractures and not directly relevant to normal excitation-contraction coupling.

Calcium Influx Related to Excitation-Contraction Coupling

The most striking result of our experiments is the existence of a component of calcium influx that occurs after a conditioning contracture and yet is closely related to excitation-contraction coupling. This influx must flow through a transport system not blocked by D600, which is present during and after the contracture in all our experiments. This flux flows at a time when the muscle is paralyzed and charge movement cannot be activated; the flux probably refills a store of calcium depleted during the prior contracture. A subsequent application of potassium (now to a paralyzed fiber devoid of charge movement) does not produce this refilling component. Depolarization in this case does not produce excitation-contraction coupling (because charge movement is blocked) and so the store of calcium is not depleted and cannot be subsequently refilled.

The refilling influx amounts to 1.3 pmol/min in 0.7 cm of a fiber of 140 μm

diam, containing ~0.6 pmol of calcium in 340 pl volume of T lumen surrounded by 0.24 cm^2 of T membrane (Mobley and Eisenberg, 1975). Thus, it corresponds to a flux of 3.8 mM/(min·liter of T volume) compared with a presumed calcium content of 1.8 mM/liter (Almers et al., 1981). In other units, the refilling influx is 12 μM/(min·liter of fiber volume), or 5.5 pmol/(min·cm^2 of T membrane), or 17 nA/(cm^2 of T membrane). The influx corresponds to 0.7 calcium ions/s for each foot process at the T-SR junction (Franzini-Armstrong, 1975, Table III), or 23 calcium ions/s for each activated pillar (Eisenberg and Eisenberg, 1982), or roughly 4 calcium ions/s for each nitrendipine binding site on the T membrane (Fosset et al., 1983*b*).

We are ignorant of the mechanism of this influx and so are not sure how reliable our estimate is of its value, particularly if some or all of the flux occurs after repolarization. A few preliminary experiments have been done (Curtis and Eisenberg, 1984) quickly rinsing away (with a flood of nonradioactive Ringer) the potassium that induced the contracture and then adding ^{45}Ca/Na Ringer. ^{45}Ca was added after the fiber was substantially repolarized and was entering its mechanically refractory period. In a contracting fiber treated this way, the calcium uptake recorded after the rinse was essentially the same as the delayed flux reported here (see Fig. 8). In a paralyzed fiber, the uptake was the same as resting. The delayed influx apparently occurs during the mechanically refractory period, the time when the ability to produce a second contracture is returning with first-order kinetics (Hodgkin and Horowicz, 1960).

Receptor-Effector Coupling

It seems likely to us that the proteins which form the mechanism of T-SR coupling in muscle fibers will be used in receptor-effector coupling in other cells. In that way, the eukaryote can efficiently use the proteins encoded by the genome common to all its cells (Alberts et al., 1983). The experiments reported here reveal further complexity and subtlety in the role of calcium in excitation-contraction coupling in skeletal muscle: other workers have shown that the calcium binding sites and calcium currents found in the T system have no obvious function; now we find that the calcium influx related to excitation-contraction coupling occurs after coupling is finished and so cannot be causally involved in that mechanism. It seems possible that the roles for messenger calcium in other receptor-effector systems will be at least as subtle, complex, and confusing as in the excitation-contraction system of skeletal muscle. Mechanisms currently proposed will probably prove as oversimplified from a molecular point of view as A. V. Hill's (1948, 1949) suggestions proved to be from a structural point of view.

T-SR Coupling

Our results demonstrate that the calcium uptake related to contracture occurs after the contracture; we find no signs of a calcium entry related to contraction before or during calcium release from the SR. Thus, one of the possible, and oft-discussed, mechanisms of T-SR coupling, namely the flow of calcium from the extracellular space ("trigger calcium": Bianchi and Frank, 1982), is ruled out in its original form. The calcium influx we observe is probably a "reflux" refilling

a binding site on the cytoplasmic side of the T membrane (Eisenberg and Eisenberg, 1982, Fig. 6), which had been emptied during the prior contracture, perhaps to initiate it. The movement of calcium from the binding site on the T membrane to another site on the SR might fulfill the functional role of the "rigid rod" of Schneider and Chandler (1973). The calcium movement might transport and transduce the charge movement signal in the T membrane to the calcium release mechanism (Fabiato, 1983) of the SR.

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