Ca Fluxes in Single Twitch Muscle Fibers

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ABSTRACT Ca influx and efflux in single twitch muscle fibers were determined by the movement of ^{45}Ca. The isotope was assayed by counting the center 1 cm of a fiber while it was in nonradioactive Rnger's solution. The average resting influx in 1.0 mM Ca Ringer's was 0.26 pM Ca/cm^2. sec for 5 to 20 min influx periods. The average additional influx upon stimulation in 1.0 mM Ca was 0.73 pM Ca/cm^2. twitch. The efflux after both resting and stimulated ^{45}Ca influx can be described by a single exponential curve with an average time constant of 125 min. This relationship is an indication of Ca exchange with a single intracellular compartment. This compartment contains an estimated 47% of the total muscle Ca at 1.0 mM Ca. When the Ca in the Ringer was reduced to 0.5 mM Ca, both the resting and stimulated Ca fluxes decreased. When Ca was raised to 1.8 mM, the stimulated influxes increased but the resting influx did not.

The movement of Ca between subcellular compartments forms an attractive hypothesis for the intracellular control of contraction in fast skeletal muscle (see Symposia in *Fed. Proc.*, 1965, **24**, 1112–1146; and 1964, **23**, 885–933). Depolarization of the surface membrane and of the transverse elements of the sarcoplasmic reticulum is thought to release Ca from some intracellular store. It is the interaction of this Ca with the contractile proteins which results in tension production. The Ca is then removed from the contractile proteins by a part of the sarcoplasmic reticulum. Hence the "activator" Ca is thought to move only within the cell.

What then is the role of extracellular Ca in contraction? To examine this point, previous investigators, most notably Bianchi and Shanes (1959), have examined Ca fluxes in whole skeletal muscle. These studies have produced many interesting results, but Ca fluxes from connective tissue and from extracellular spaces have complicated their interpretation. The aim of the experiments reported here is to avoid these difficulties by examining the Ca fluxes from the center portion of a single muscle fiber.

METHODS

Muscle Fibers Single twitch muscle fibers were isolated from the semitendinosus muscle of *Rana pipiens*. The width of each fiber was measured at ten places along

its length to obtain a value for surface area. For the flux calculations the fiber was considered to be cylindrical.

Solutions The standard Ringer solution had a pH of 7.0–7.2 and contained (in mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.0; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85. All solutions were filtered through a fine sintered glass filter to remove dust. Suitable quantities of 1 M EDTA solution were added, when indicated, to the stock Ringer to obtain a final concentration of 10 mM. Hypertonic solutions contained an additional 165 mM of NaCl.

The radioactive ^{45}Ca Ringer was made by evaporating a sample of $^{45}CaCl_2$ (^{45}Ca–P–3; Union Carbide Corp.) to dryness, dissolving it in distilled water, redrying, and finally dissolving it in Ca-free Ringer's. The carrier Ca in the $^{45}CaCl_2$ sample provided the 1.0 mM Ca in the final ^{45}Ca Ringer. The activity of the ^{45}Ca solution was about 1 mc/ml. The solution was filtered through a 0.8 μ Millipore filter to remove some unknown particulate matter, containing ^{45}Ca, which stuck to the fibers and greatly increased the apparent influx.

The Experimental Chambers The general design of the influx and counting chambers is similar to those used by Hodgkin and Horowicz (1959) and Keynes (1951) and is depicted in Fig. 1. The forceps were attached to an overhead mechanism which permitted the fiber to be moved about rapidly. All experiments were done at room temperature (18–20°C).

The counting window was machined to a width of 1 cm. The effective counting width was determined by placing a piece of 80 μ Intermedic tubing, partially filled with a concentrated ^{45}Ca solution, 20 μ above the Mylar window. After determining the counting rate and the position of the meniscus relative to the end of the window, the tube was moved horizontally to reduce the column of ^{45}Ca over the window and the counting rate was redetermined. A plot of the length of the column of ^{45}Ca over the window vs. counting rate showed that the rate diminishes linearly with distance and reaches background level 250 μ beyond the edge of the window. Since the error in measurement might well be ± 250 μ, the effective counting width was considered to be 1 cm.

General Procedure The fiber was mounted and stretched to $\frac{4}{3}$ of its slack length and then stimulated electrically to check for excitability. The appearance of an all-or-nothing response at both ends of the fiber indicated an excitable fiber. The condition of the fiber was checked several times during the experiment by stimulation and also by visual observation. If the fiber was excitable 1 hr after mounting, it was transferred to the influx cell and lowered almost to the bottom of the trough. A drop of ^{45}Ca Ringer's was added to the Ringer in the influx trough and after a predetermined time the cell was flooded with inactive Ringer's. The fiber was then transferred to the counting chamber and placed level with the window about 20 μ above it. The position was carefully measured with a horizontal dissecting microscope so that the fiber could be returned to this exact vertical position. This precaution was necessary since it was observed experimentally that a vertical movement of 47 μ altered the counting rate by a factor of 2. The fiber's vertical position was checked periodically during the efflux periods. It was not uncommon for the fiber-counting rate to increase unex-

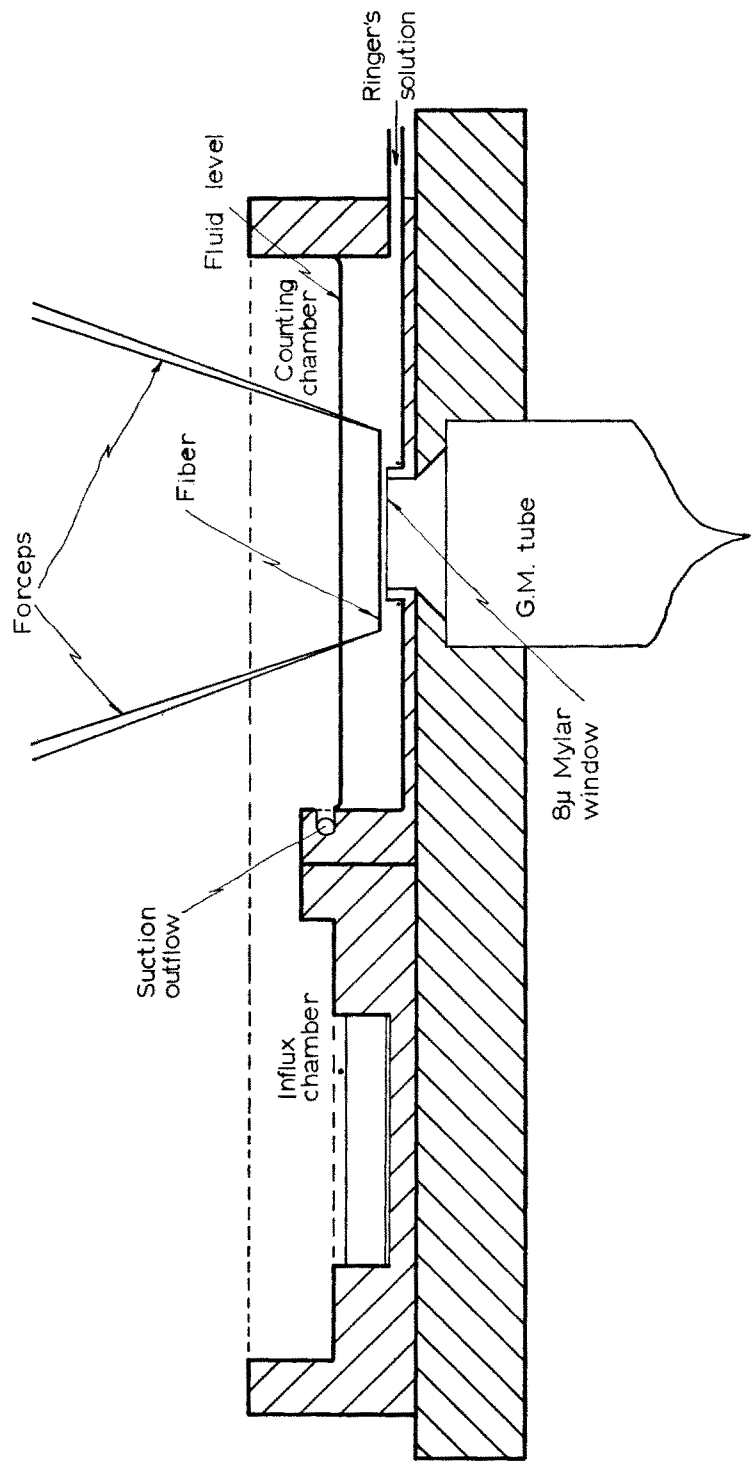

FIGURE 1. A simplified diagram of the apparatus. The chambers were machined out of Lucite and were connected by a narrow channel so that both chambers could be flooded and the fiber transferred from one to the other under fluid. The front face of the counting chamber was a glass microscope slide which allowed observation of the vertical position of the fiber with a high-power dissecting microscope. Both chambers contained a pair of stimulating electrodes. The trough in the bottom of the influx chamber was made of glass tubing split lengthwise.

pectedly and in each case it was observed that the fiber had moved closer to the window. Such a case is shown in Fig. 5. Upon discovery, the fiber was returned to the original position and the erratic counts discarded. This possibility of movement made efflux periods greater than 2 hr. impractical.

At the close of the experiment the fiber was raised above the Ringer solution and the center 1 cm of fiber was cut out and placed onto a planchet together with 5 drops of detergent solution. This planchet and diluted samples of the soak-in solutions were individually counted by a thin-window G-M tube. All planchets contained an equal weight of salt to keep salt absorption constant since the method depends on equal counting efficiency of the ^{45}Ca in both the sample of soak-in solution and the fiber. The sensitivity of the G-M tube in the efflux chamber was calculated from the following equation:

$$\frac{\text{CPM in the fiber on the planchet}}{\text{CPM in the fiber in the efflux chamber}} \times \frac{\text{Moles Ca in the sample of soak solution}}{\text{CPM in the sample of soak-in solution on the planchet}}$$

Since 1 cm of fiber is counted in both the efflux chamber and on the planchet, the sensitivity was in terms of moles Ca/CPM/cm of fiber and was approximately 10^{-13} moles Ca/CPM/cm.

The influx was calculated from the formula (Hodgkin and Horowicz, 1959):

$$\frac{\text{CPM taken up/time in } ^{45}Ca \times \text{sensitivity}}{\pi(\text{diameter})\left(1 - \frac{\text{time in } ^{45}Ca}{2 \times \text{time constant for efflux}}\right)}$$

After the center of the fiber had been cut away, the forceps were lowered to the counting position and the counting rate determined. This rate was always the same as the background counting rate taken with the forceps raised well above the counting chamber. This indicates once again that the G-M tube was not sensitive to radiation from a source in the position of the tendons. Occasionally the tendons were placed on a planchet and counted with the thin-window G-M tube. They had a counting rate about ten times greater than the fiber-counting rate.

All counting rates were corrected for background-counting rate before use or inclusion in figures. Counting rates were not corrected for isotope decay. Since the half-life of ^{45}Ca is 164 days, this correction was not significant for an experiment lasting one-half day.

RESULTS

Resting and Stimulated Ca Influxes in 1.0mM Ca Ringer's Fig. 2 shows both resting and stimulated Ca influxes. After a 15 min influx the muscle was transferred to the counting chamber. Counting started 2.5 min after the end of the ^{45}Ca influx period and the counts were of 5 min duration. After 70 min the muscle was moved back to the influx chamber and stimulated while in the

^{45}Ca solution. The effect of the stimulation was observed visually and a brisk twitch noted for each stimulus. After 5 min of stimulation (200 stimuli) the chamber was flooded with Ringer's solution to end the influx period.

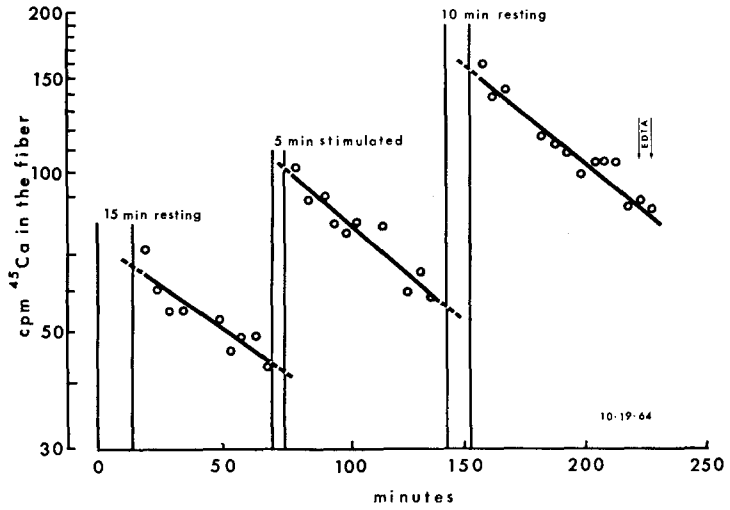

FIGURE 2. Resting and stimulated Ca fluxes in 1.0 mM Ca Ringer's. The fiber was exposed to ^{45}Ca during the periods indicated by the vertical bars. After each influx the muscle was placed over a G-M tube and counted while inactive Ringer's flowed past the fiber. At the end of the experiment the fiber-counting rate was 80 CPM. The center 1 cm counted 148 CPM on a planchet, or 1 chamber CPM = 1.85 planchet CPM. A diluted sample of the first soak-in solution, containing 2×10^{-10} moles Ca, counted 5902 CPM on a similar planchet. The sensitivity of the G-M tube in the counting chamber is therefore (2×10^{-10} moles Ca/5902 CPM) (1.85/1 cm of fiber) = 6.3×10^{-14} moles Ca/CPM/cm. The specific activity of the soak-in solution varied somewhat from influx to influx. The other two sensitivities were: 7.6×10^{-14} moles Ca/CPM/cm and 5.8×10^{-14} moles Ca/CPM/cm. The average fiber diameter was 76 μ. The resultant influxes were: first resting = 0.20 pM Ca/cm^2·sec; second resting = 0.44 pM Ca/cm^2·sec; stimulated = 0.44 pM Ca/cm^2·twitch. The Ca influx per twitch was calculated by subtracting the average resting influx from the stimulated influx and dividing by the total number of stimuli. The time constants for efflux, as fitted by least square analysis, were 144, 110, and 114 min.

The fiber was moved back to the counting chamber and counted again at 5 min intervals. After a 60 min period the fiber was moved back to the influx chamber for a second resting influx followed by an efflux period. It can be seen that the time constant for efflux was approximately constant during the three efflux periods. It can also be seen that the Ca influx per minute is greater when the muscle is stimulated.

The Effect of the Fiber Sticking to Glass Early in this experimental series, almost all the Ringer solution in the influx trough was removed before the

^{45}Ca Ringer was added. This caused the fiber to stick to the glass bottom of the influx trough. At the end of the influx period this ^{45}Ca Ringer's was removed and saved for future experiments before the influx chamber was flooded with Ringer's. A very disturbing feature of these experiments was an increase in the time constant for efflux after an influx period. Fig. 5 shows this behavior.

Why the time constant for efflux should increase when the fiber is stuck onto glass is not clear, but the effect can be produced if the maneuvers in the influx chamber are carried out with inactive Ringer's. This increase in the efflux time constant occurs after the first influx period as well. In fibers so treated, the average time constant for efflux was 340 min (ten fibers). This time constant is much greater than the average time constant (125 min) for efflux from fibers not stuck to glass. The average value for resting influx in fibers so treated was 0.11 pmCa/cm^2·sec and for stimulated influx 0.15 pmCa/cm^2·twitch. These values are considerably lower than those given in Table I.

The Sticky Precipitate In the Methods section an unknown sticky precipitate containing ^{45}Ca was mentioned. Several times in the course of this investigation, the resting influx of ^{45}Ca was larger than expected; Fig. 3 is a good example. The first influx gave an apparent value of 0.4 pM/cm^2·sec and t_c of 360 min. When the normal Ringer flowing past the fiber was changed to 10 mM EDTA Ringer's for 5 min, the counting rate declined rapidly and after 3 min a second straight line was used to calculate an influx of 0.03 pM/cm^2·sec with t_c equal to 100 min. The ^{45}Ca solution was then filtered and a second resting influx was done on the same fiber. The influx was 0.01 pM/cm^2·sec and t_c = 150 min. When 10 mM EDTA was added to the Ringer neither the counting rate nor t_c was altered. Ringer's was again substituted. The fiber was permanently inexcitable.

The fibers varied in their sensitivity to 10 mM EDTA Ringer's; some were inexcitable after 5 min, while others withstood a 10 min exposure with no apparent ill effects. The application of the 10 mM EDTA solution was occasionally followed by a contracture. At the end of several of the apparently normal efflux experiments presented in Table I, the fiber was treated with 10 mM EDTA Ringer's solution for 5 min and no effect on counting rate or t_c was observed. This suggests that the results presented in Table I were not significantly affected by this precipitate. It is concluded that any ^{45}Ca efflux caused by the application of EDTA does not originate from the surface or the interior of the muscle fiber.

Resting and Stimulated Ca Influxes in 0.5 and 1.8mM Ca Ringer's The Ringer solution used in these experiments contained a total of either 0.5 or 1.8 mM Ca in addition to the other constituents given in the Methods section. The format of the experiments was the same as shown in Fig. 2. The results of these experiments are tabulated in Table I.

TABLE I

Ca IN FLUXES

Fiber	Diameter (μ)	Resting (pM/cm²·sec)	Stimulated (pM/cm²·twitch)	Time constant for efflux (min)
		0.5 mM Ca Ringer's		
2.9.65	96	0.02	0.12	50
		0.02		
2.11.65	67	0.05	0.02	42
2.17.65	77	0.06	0.60	146
		0.14		
2.22.65	56	0.14	0.50	94
		0.25	0.45	
Average $\pm$ $s/\sqrt{n}$		0.10±0.03	0.34±0.09	83
		1.0 mM Ca Ringer's		
9.19.64	81	0.22		210
9.21.64	65	0.05		111
10.12.64	70	0.26	0.34	169
10.19.64	78	0.20	0.44	121
		0.44		
10.23.64	88	0.23	1.00	60
			1.12	
10.28.64	56	0.28	0.83	56
			0.69	
11.27.64	85	0.24		144
12.7.64	69	0.48		53
Average $\pm$ $s/\sqrt{n}$		0.26±0.02	0.73±0.12	125
		1.8 mM Ca Ringer's		
12.16.64	74	0.09	0.82	158
		0.42		
12.18.64	99	0.13	3.05	124
		0.62		
1.25.65	64	0.12	0.08	225
		0.35		
1.29.65	60	0.12	0.20	155
		0.14		
Average $\pm$ $s/\sqrt{n}$		0.25±0.07	1.04±0.6	165

From the average values in Table I it can be seen that a linear relationship exists between the stimulated influx and Ca concentration of the external solution. The resting influx, on the other hand, decreases below 1 mM Ca but is constant above 1 mM Ca.

The average resting and stimulated influxes in 1.0 mM Ca agree fairly well with the values for sartorius muscle given by Bianchi and Shanes (1959).

Shanes and Bianchi (1960) give 0.094 pM Ca/cm²·sec as the average resting influx and 0.20 pM C/cm²·twitch as the stimulated influx.

The Effect of a Resting Influx Preceding a Stimulated Influx In all the preceding experiments stimulation of the muscle began very soon (2 to 3 sec) after the ^{45}Ca was placed around the fiber. If, as suggested by Bianchi and

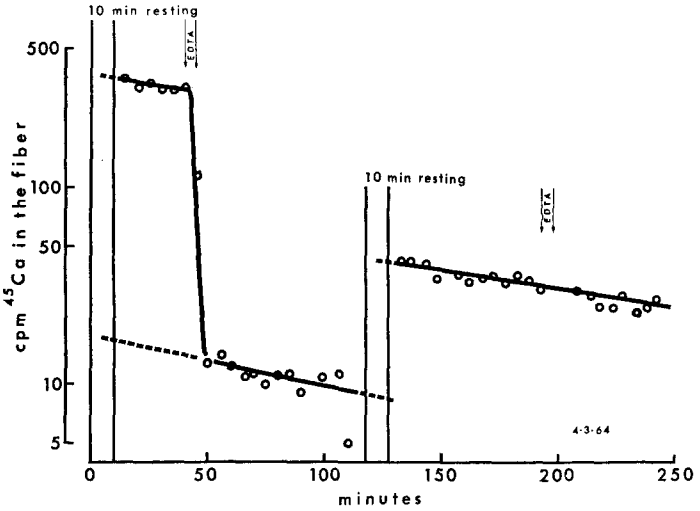

FIGURE 3. The effect of filtering stored ^{45}Ca Ringer's solution upon the apparent influx. The ^{45}Ca solution used in the first influx period had been stored in the cold for some time. The apparent influx was 0.4 pM Ca/cm²·sec. When the fiber was treated with 10 mM/EDTA in Ringer's, a large fraction of the Ca was very rapidly removed. When the new line was extrapolated to zero, the apparent influx was 0.03 pM Ca/cm²·sec. The ^{45}Ca Ringer was filtered through a 0.8 μ Millipore filter before being used in the second influx. The filtering changed the specific activity. The influx was 0.01 pM Ca/cm²·sec. The time constants for efflux are 360, 188, and 245 min. The fiber stuck to the glass influx trough before and after the influx period. The fiber diameter was 83 μ.

Shanes (1959), the ^{45}Ca must be fixed to, or exchanged with, some surface site before entering the fiber, then this procedure should lead to fallaciously low values for stimulated influx. To test this point, two stimulated influxes separated by a resting influx were done. The first stimulated influx was brief, with stimulation beginning as the isotope was applied. The second stimulation began several minutes after the isotope was placed around the fiber. In experiments on two fibers (10.23, 10.28) both stimulated influxes were the same.

It can be concluded that "surface" site exchange must either be very rapid or absent under these conditions. It should be noted that Bianchi and Shanes' (1959) conclusion was based on a Ca influx into whole muscle during contracture. It is quite possible that the increase resulting from presoaking their muscles was the result of prolonged diffusion time in the extracellular spaces.

Time Constant for Efflux The efflux of ^{45}Ca from single muscle fibers after short exposure to ^{45}Ca can be described adequately by a single exponential curve. This is true of all the fibers examined. Occasionally the first point of the efflux curve would be considerably, and probably significantly, above the best straight line through the remaining points. The first efflux curve in Fig. 2 is an example of this. The first points of the following effluxes were not significantly above the line and this was generally the case. Consequently these high points cannot be used as evidence for a rapid second component of the curve.

Two single fibers were left in the ^{45}Ca Ringer for 30 min and were then counted at 1 min intervals for the first 10 min of efflux and thereafter at 5 min intervals for 2 hr. The best straight line through the 5 min points also described the 1 min points, although the scatter in the early points was, of course, much greater because of the shorter counting interval.

All the efflux data strongly suggest a single exponential relationship between ^{45}Ca loss and time, which in turn suggests a single barrier which limits the rate of Ca movement to the external environment. This barrier is presumably a membrane and delimits an intracellular compartment.

What fraction of the total intracellular Ca is contained within this compartment? For reasons discussed in the introduction, it is fairly clear that Ca is not evenly distributed throughout the resting muscle fiber. While the amount of Ca in a fiber can be expressed in terms of moles of Ca/liter of fiber, it cannot be expressed as a concentration since the volume it occupies is unknown. The Ca in the exchangeable compartment can be calculated from the value of resting influx, the time constant, and the fiber diameter. Following the derivation of Hodgkin and Horowicz (1959, pp. 416–17) $Ca_i = M_{in}\tau S/V$. This equation is valid if: (*a*) the volume of the fiber is constant; (*b*) the movements of Ca are limited by a surface membrane; (*c*) the Ca in the compartment is rapidly mixed; and (*d*) M_{in} and τ are constant for periods long compared to τ. From the last condition it follows that the Ca concentration of the compartment is constant and that the influx equals the efflux.

Since all these conditions are fairly well met in these experiments, the Ca in the compartment was calculated and expressed as millimoles of Ca per liter of fiber. These results are presented in Fig. 4 together with analytical values for total muscle Ca. It is apparent that the Ca in the exchangeable compartment and in the whole muscle decreases with decreasing Ca.

Since the exchangeable and total Ca decrease roughly in parallel, the non-exchangeable Ca is apparently unaffected by decreasing extracellular Ca. When Winegrad's (1965) data for total toe muscle Ca (1.93 μmole Ca/g muscle or 1.87 mM Ca/liter muscle) are used, the exchangeable compartment contains 47% of the total fiber Ca at 1.0 mM extracellular Ca.

The Effect of Cardiac Glycosides upon Ca Fluxes As a matter of curiosity, I investigated effects of the cardiac glycosides, ouabain and strophanthidin,

upon Ca fluxes. These compounds inhibit the active transport of Na and K in a number of tissues. In experiments on two fibers neither 10^{-5} M ouabain nor 10^{-4} M strophanthidin had any effect on the resting influx or the time constant for efflux.

Hypertonic Solutions The results of the foregoing sections have confirmed previous observations (Bianchi and Shanes, 1959) that the Ca influx increases during stimulation. The series of experiments in this section was done to rule out any effect of muscle movement during contraction upon Ca influx.

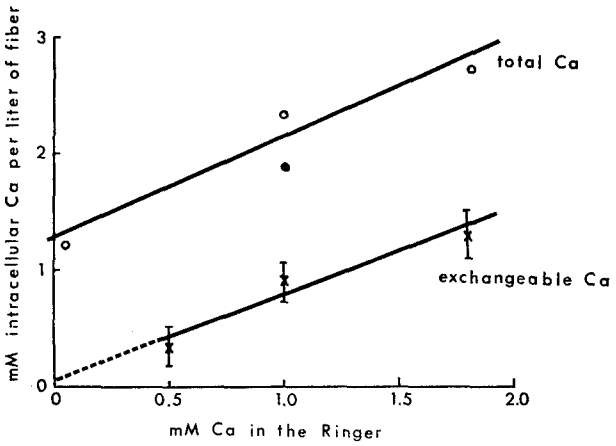

FIGURE 4. The relationship between total and exchangeable Ca and the Ca concentration of the Ringer solution. The values for the exchangeable Ca (X) $\pm s/\sqrt{n}$ are calculated from influx data and time constant for efflux. The open circle values for total Ca are from Gilbert and Fenn (1957) and the closed circle value is from Winegrad (1965).

When a muscle is stimulated in hypertonic solution, an action potential but no concomitant twitch is observed (Hodgkin and Horowicz, 1957). An increased resistance to stretch following stimulation (Howarth, 1958) strongly suggests that the active state is set up in muscles in hypertonic solution. The hypertonic solution apparently prevents the muscle from contracting even though force is being produced by the actomyosin contractile system.

Many authors have suggested that the actomyosin contractile system is activated by Ca. If the observed increased Ca influx is associated with activation rather than movement, then the stimulated Ca influx might be expected to be unaffected by hypertonic solutions.

The effect of hypertonic solution on resting and stimulated influx in 1.0 mM Ca Ringer's is shown in Fig. 5. The format of the experiment is similar to that shown previously in Fig. 2 except that the fiber was placed in hypertonic Ringer's for 5 min before the influx in hypertonic ^{45}Ca began. The efflux was

into Ringer's solution. The average data are: Resting influx = 0.15 ± 0.03 pM Ca/cm²·sec; stimulated influx = 0.11 ± 0.05 pM Ca/cm²·twitch. There are six and four determinations respectively. Since the fibers in these experiments were stuck to the influx trough, these data should be compared with the average data from fibers stuck to the glass influx trough. Those values are:

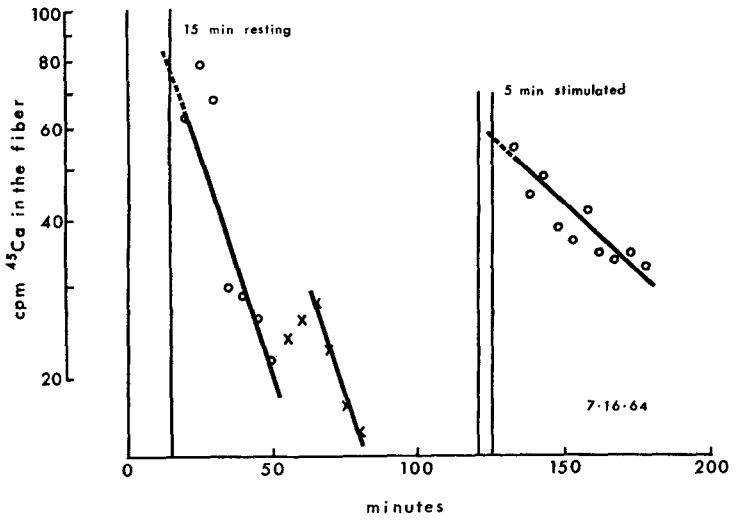

FIGURE 5. The effect of hypertonic solution upon resting and stimulated Ca influxes. The fiber was placed in hypertonic solution before each influx in hypertonic ^{45}Ca Ringer's. The efflux was into Ringer's. The counting rate slowly increased after 35 min of the first efflux and then resumed a steady fall parallel to the initial straight line. When the fiber position was checked, it was found to be considerably closer to the counting window. During stimulation in the hypertonic Ringer the ends of the fiber were observed to move very slightly. The influxes were: resting, 0.18 pM Ca/cm²·sec and stimulated, 0.12 pM Ca/cm²·twitch. The time constants were 28 and 102 min. The fiber diameter was 73 μ in Ringer's, 58 μ in hypertonic Ringer's. The fiber was stuck to the glass influx trough before and after each ^{45}Ca influx period.

resting influx = 0.11 pM Ca/cm²·sec and stimulated influx = 0.15 pM Ca/cm²·twitch. It will be noted that the values are unchanged by the hypertonic Ringer. The fiber diameters quoted are the diameters in hypertonic solution which are 80% of the diameter in Ringer's.

DISCUSSION

Perhaps the most striking feature of the experiments described here is that ^{45}Ca efflux from a muscle fiber soaked for a short time in ^{45}Ca can be described by a single exponential curve. This strongly suggests that the ^{45}Ca is exchanging with a single intracellular compartment. This compartment contains approximately one-half of the total fiber Ca when the extracellular Ca is 1.0 mM.

From an analysis of ^{45}Ca influx data, Gilbert and Fenn (1957) calculated that 39% of the intracellular Ca is exchangeable. Shanes and Bianchi (1959) calculated that 38% is exchangeable. Harris (1957) calculated that 10 to 25% of the muscle Ca exchanged after 16 hr at 4°C. Since this figure was obtained at such a low temperature, it will be discounted. From the data given by Winegrad (1956, Table I), it can be calculated that 44% of the total toe muscle Ca has exchanged after 480 min. The agreement between these figures obtained under conditions near equilibrium and the data presented in this paper is very gratifying.

The data presented here disagree with the data of Bianchi and Shanes in two minor points: (*a*) The resting influx in 1.8 mM Ca is no larger than in 1.0 mM Ca. They present evidence that the influx in three times normal Ca is higher than in normal Ca. It is possible that the influx increases between 1.8 and 3.0 mM Ca. It is also possible that the high figure for 3.0 mM Ca results from the "sticky precipitate" described under Results. (*b*) There is no evidence for a surface-bound Ca phase. It is quite possible that the phenomena Bianchi and Shanes ascribed to a bound surface phase were caused by diffusion delays and/or binding to some nonmuscle fiber component of the sartorius muscle.

The average time constant for efflux into 1.0 mM Ca (124 min) is considerably shorter than the 500 min time constant given by Shanes and Bianchi (1959). Before discussing this difference in the time constants, some more recent results should be mentioned. These results can only be considered as preliminary but have been presented at the 1965 annual meeting of the Society of General Physiologists.

I have designed a chamber which collects the effluent from only the center 1 cm of the length of a single muscle fiber or a bundle of muscle fibers. After a 4 hr influx, the efflux can be described by a two-component semilog curve. The first component has an average time constant of 57 min (six bundles). The second time constant is much longer, 600 to 800 min. At present all that can be said about these results is that the first time constant clearly corresponds to the single time constant described in this paper for efflux after a short influx. It is fairly clear that Shanes and Bianchi are describing the second of these components and that the first was lost in the initial efflux phase.

I would like to thank Dr. Paul Horowicz for the kind hospitality of his laboratory. I am most grateful for his very helpful discussion as well as that of Drs. R. H. Adrian and L. E. Moore.

I would also like to thank the National Science Foundation for a Postdoctoral Fellowship which made this research possible.

Received for publication 25 January 1966.

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