The Effect of Caffeine on Radiocalcium Movement in Frog Sartorius

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ABSTRACT Caffeine increases resting calcium influx approximately threefold in normally polarized and in potassium-depolarized fibers of frog sartorius muscles. It does not affect the transient rapid increase in calcium influx that occurs at the beginning of a potassium depolarization. Calcium outflux in Ringer's solution, in zero calcium Ringer's solution, and in zero calcium Ringer's solution plus 0.004 M EDTA is also markedly increased by caffeine. The increased outflux reaches a rate which is approximately the same as the increased calcium influx. One interpretation of the findings is that caffeine reduces the binding of calcium both in the membrane and in the myoplasm; this increases the "permeability" to calcium and the ionic activity of calcium in muscle. This interpretation is consistent with the view that the contractile state of muscle is dependent at least in part on the thermodynamic activity of calcium in the muscle fibers.

Experiments by Axelsson and Thesleff (1) suggested that the alkaloid caffeine (free base) initiates a process in the muscle which leads to contraction, and that this process is not mediated by changes in resting potential or in ionic permeability of the muscle membrane. The addition of Ca up to five times its normal concentration or the depletion of Ca by soaking the muscle in 0.1 per cent ethylenediaminetetraacetic acid (EDTA) has no observable effect on the contracture produced by caffeine. Calcium has been suggested as a possible link between depolarization and contraction (6, 9, 13, 3), a suggestion supported by recent findings (2, 15). The following experiments were performed to determine whether caffeine might still be exerting its effects by way of the calcium in the muscle, especially that remaining after EDTA treatment (5).

METHODS

Ca^{45} uptake in frog sartorius was measured by a procedure given in a previous paper (2). Paired frog (*Rana pipiens*) sartorius muscles were placed for a period of 5 or 10 minutes in radiocalcium (0.5 μc./ml.) Ringer's solution containing either no caffeine or 0.005 M caffeine; after exposure the muscles were removed and the extracellular radiocalcium washed out by repeated soaking at frequent intervals (5, 10, 25, and 50 minutes) in a series of test tubes containing 5 ml. of Ringer's solution. The tissues were ashed after 90 minutes' washout and the radioactivity of the acid extract of the residue determined. A correction factor for the amount of calcium lost from the slow component during washout was obtained from the average time constant for the slow component, which for the muscles used in these experiments was 254 minutes. The amount of radioactivity at the end of 90 minutes' washout was therefore increased by a factor of 1.42.

The Ringer's solution used consisted of 1 mM $CaCl_2$, 1.6 mM KCl, 108 mM NaCl, and 0.95 mM all sodium-Sørensen phosphate buffer. Occasionally sartorius muscles will spontaneously twitch in 1 mM Ca which reflects itself in an erratic outflux curve. The occasional spontaneous twitching was obviated by the addition of 2 mg. per cent cocaine to Ringer's solution and 14 mg. per cent cocaine if calcium was omitted. When 0.004 M EDTA was added to Ringer's solution, the pH was brought back to 7.1 by addition of HCl.

The calcium uptake was determined by calculating the Ca^{45} space (milliliters per gram) of the muscles and converting to calcium uptake per gram by multiplying the space by the calcium concentration of Ringer's solution. In order to compute the fluxes the calcium uptake per gram was divided by the surface area of the fibers which was estimated to be 300 $cm.^2/gm.$ (2).

Ca^{45} uptake in muscles depolarized with 0.080 M KCl was measured in two ways: (*a*) the muscles were depolarized for 5 minutes in high K^+–Ringer's solution prior to addition of high K^+–Ringer's solution with radiocalcium and 0.005 M caffeine; (*b*) radiocalcium Ringer's solution was applied to the muscle for 2 minutes prior to the addition of K^+–Ringer's solution with Ca^{45} and caffeine. The first procedure eliminated the Ca^{45} uptake due to the initial potassium depolarization and contracture; the second included the initial uptake during K^+ depolarization plus the uptake during the maintained depolarization. The contractures observed under these conditions were all recorded isometrically.

The outflux studies were performed by presoaking the sartorii or Achilles tendons, mounted on glass rods, for 4 hours in oxygenated radiocalcium Ringer's solution, removing the muscles, dipping them rapidly in non-radioactive Ringer's solution, and blotting them uniformly on Whatman No. 5 filter paper. The muscles were then placed in an automatic collection apparatus which circulates a 2 ml. column of oxygenated Ringer's solution past the muscle or tendon and collects the solution in a planchet at preset time intervals of 5 minutes for the first two collections and 10 minutes for the remaining collections. The Ca^{45} of each collection and of the muscle or tendon at the end of the washout period was determined; from these data "desaturation" and "rate coefficient" curves were constructed (14). The former describe the decline of tissue radioactivity as per cent of initial tissue radioactivity with time. The

latter is the time course of the average per cent change in activity per minute which is estimated for each collection interval by dividing the activity appearing in the medium by the mean activity in the tissue during the collection interval and by the duration of the collection interval.

These experiments were performed from May to October, and were carried out at a room temperature of 25°C.

RESULTS

Effect of Caffeine on Contraction and Contracture

Preliminary investigation showed that caffeine concentrations of 0.005 M or less increased the muscle twitch height but caused no observable contracture for periods up to 5 minutes; if the muscles were depolarized by adding 0.080 M KCl to Ringer's solution, the potassium-induced contracture was increased in strength and lasted as long as caffeine was present. In the control muscles without caffeine, the potassium-induced contracture spontaneously relaxed after 1.5 to 2.5 minutes in the continued presence of elevated potassium. The caffeine concentration of 0.005 M was chosen for influx and outflux studies of radiocalcium because its effects on muscle contraction were reversible for the short periods of equilibration used in these experiments.

The frogs employed were not so sensitive to caffeine as those used by Axelsson and Thesleff (1), where graded caffeine contractures could be obtained in caffeine concentrations of 1.5 to 5.0 μmoles/ml. within 10 seconds. In December, after the experiments reported here were finished, the sensitivity of frog sartorius to caffeine was again examined, and it was found that the sensitivity had increased; no contractures were seen below 0.005 M caffeine after 5 minutes' exposure, but a contracture did occur after 3 minutes' exposure to 0.005 M caffeine, which was only slowly reversible upon removal of caffeine.

Effect of Caffeine on Ca^{45} Influx during Rest and Potassium Treatment

The influx data and standard errors of the means are tabulated in Table I. In row A the average resting influx of radiocalcium for the muscles used in these experiments based on a 5 or 10 minutes' exposure to Ca^{45} is seen to be 0.096 $\mu\mu$mole/cm.^{2} sec., which can be compared to the values of 0.094 and 0.072, for a 10 and 60 minute exposure, respectively (2). The addition of 0.0025 M caffeine to the Ca^{45} Ringer's solution more than doubles the rate of calcium influx; 0.005 M caffeine nearly triples the rate of influx. Under these conditions no detectable contractures appear; but in the presence of 0.005 M caffeine, the potassium-induced contractures are maintained until the caffeine is removed after 3 minutes. Control muscles, in the absence of

TABLE I

CALCIUM INFLUX IN FROG SARTORIUS MUSCLE

Row	No. of Experiments		Conditions	Influx	Experimental-control
				μμmole/cm.² sec.	*μμmole/cm.² sec.*
A	14	Control	5 or 10 min. in Ca^{45} Ringer's	0.096 ± 0.018	
	6	Experimental	10 min. in Ca^{45} Ringer's + 0.0025 M caffeine	0.201 ± 0.037	0.105 ± 0.033(P = 0.05)*
	8	Experimental	5 or 10 min. in Ca^{45} Ringer's + 0.005 M caffeine	0.255 ± 0.042	0.157 ± 0.051(P = 0.05)*
			5 min. in Ringer's + 0.080 M KCl then:		
B	6	Control	10 min. in Ca^{45} Ringer's + 0.080 M KCl	0.072 ± 0.014	
	6	Experimental	10 min. in Ca^{45} Ringer's + 0.080 M KCl + 0.005 M caffeine	0.243 ± 0.033	0.171 ± 0.028(P = 0.01)*
			Contracture	μmole × 10^{-3}/gm.	μmole × 10^{-3}/gm.
			2 min. in Ca^{45} Ringer's then:		
C	6	Control	3 min. in Ca^{45} Ringer's + 0.080 M KCl	18.47 ± 2.9	
	6	Experimental	3 min. in Ca^{45} Ringer's + 0.080 M KCl + 0.005 M caffeine	26.3 ± 3.1	7.6 ± 1.7(P = 0.01)*

* P is based on the S.E. of differences from paired muscles.

caffeine, all spontaneously relax within 3 minutes in the high potassium Ringer's solution.

In row B it can be seen that 0.005 M caffeine, added to a potassium-depolarized muscle that has relaxed, increases the calcium influx approximately threefold, and yet no contracture occurs. The increase is about the same as occurs when caffeine is added to the regular Ringer's solution. Therefore, potassium depolarization does not alter the effectiveness of caffeine in increasing calcium influx. The calcium uptake when 0.005 M caffeine is added with potassium to muscle is increased by 40 per cent; *i.e.*, 7.6×10^{-3} μmole/gm. The 40 per cent increase can be accounted for by the increase in influx during maintained potassium depolarization rather than by an increase in the amount of calcium that enters during initial potassium depolarization. The calcium that enters during the initial period of potassium depolarization may be estimated in the following manner:

The amount of calcium that enters both the control and experimental muscles (row C, Table I) during the 2 minutes in Ca^{45} Ringer's is assumed to enter at the resting rate of 0.096 $\mu\mu$mole/cm.^{2} sec.; *i.e.*, 28.8 $\mu\mu$mole/gm. sec. By multiplying the resting rate by 120 seconds, the estimated influx during the first 2 minutes is found to be 3.5×10^{-3} μmole/gm. for both the control and experimental muscles. The calcium that enters during the 3 minutes in high potassium Ringer's solution can be determined from the corresponding influx rates given in row B. The calcium penetrating during this period is 3.9×10^{-3} μmole/gm. for the controls, and 13.1×10^{-3} μmole/gm. for the experimental muscles. The calculated resting influx is the sum for the two periods; *i.e.*, 7.4×10^{-3} μmole/gm. for the controls, and 16.6×10^{-3} μmole/gm. for the experimental preparation. Substracting the calculated resting influx from the total taken up (row C), we obtain an approximation of the amount that went in during the initial period of depolarization. This amounts to 11.3×10^{-3} for the controls and 9.7×10^{-3} μmole/gm. for the experimental muscles. Although some error is involved in the above estimation due to the short periods of exposure to Ca^{45}, the additional uptake while the space is being emptied during washout appears to compensate for the error, as shown by comparison of our 60 minute influx values (0.072 $\mu\mu$mole/cm.^{2} sec.), and 10 minute influx values (0.094 $\mu\mu$mole/cm.^{2} sec.) in our first paper (2).

Thus caffeine has little or no effect on the amount of calcium that enters during the initial potassium depolarization; moreover, potassium depolarization does not alter the enhancement of Ca^{45} entry by caffeine.

Effect of 0.005 M Caffeine on Ca^{45} Outflux in Ringer's Solution

The desaturation curve, obtained by washing a frog sartorius muscle previously soaked for five hours in Ca^{45} Ringer's solution, is plotted in Fig. 1. The corresponding curve for the rate coefficient, calculated from the de-

saturation curve, is also given. The increase in Ca^{45} efflux produced by 0.005 M caffeine added after 220 minutes is evident. The rate coefficient is seen to reach a maximum during the second 10 minute collection period and then

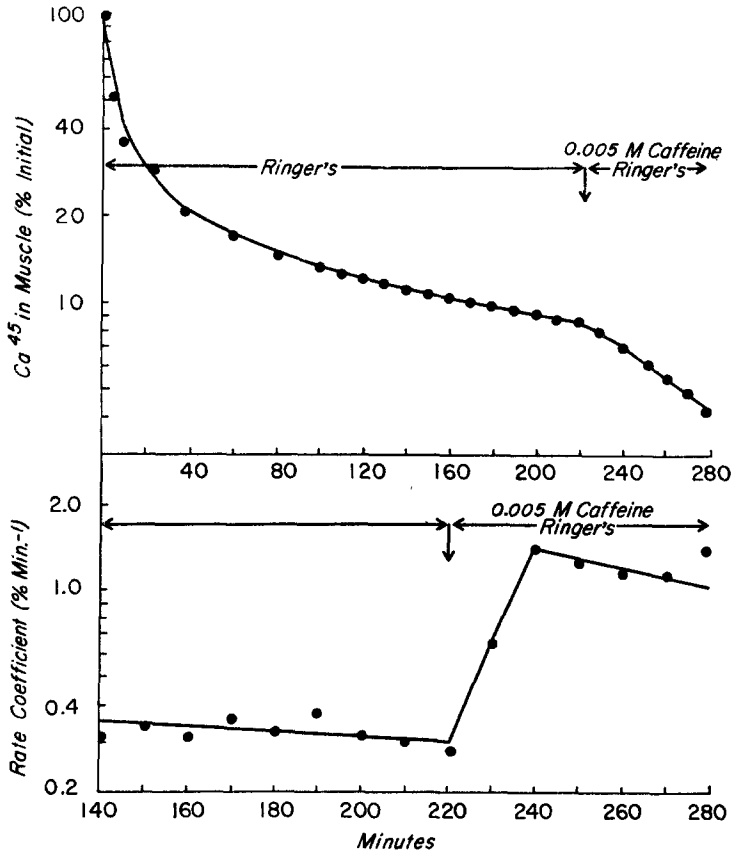

FIGURE 1. (*a*) (upper curve) Time course of the decline of Ca^{45} content of an individual muscle in Ringer's solution containing 1 mM/liter of calcium, after prior exposure for 5 hours to Ringer's solution containing Ca^{45}. At 220 minutes 0.005 M caffeine is added to the Ringer's solution. (*b*) (lower curve) Time course of the decline of the "rate coefficients" obtained from the same preparation as in upper curve. The rate coefficients from 140 minutes to 280 minutes are plotted. At 200 minutes the addition of 0.005 M caffeine causes a sudden marked increase in the rate coefficient, which reaches a maximum at 240 minutes. The increase in the rate coefficient is maintained in the continued presence of caffeine.

remains elevated. From these data the outflux can be calculated for comparison with influx.

In order to calculate the outflux of calcium from the rate coefficients the following equation was used:

Ca outflux = ("rate coefficient") (exchangeable Ca in muscle fibers)

From calcium exchange studies, the exchangeable calcium has been estimated to attain 0.33 μmole/gm. in 4 hours; from this complete exchange was further estimated to involve 0.85 μmole/gm. (14). But in our present study the average exchangeable calcium was found to be 0.22 ± 0.02 μmole/gm. at the end of 4 hours and the average time constant as determined from the outflux curves was 254 minutes for the muscles in row A, and 168 minutes for the muscles in row B of Table II, instead of 500 minutes previously obtained. The exchangeable calcium for these studies would therefore be 0.37 μmole/gm. for the muscles in row A, and 0.29 μmole/gm. for the muscles in row B of Table II.

The average rate coefficient for six collection periods prior to the addition of caffeine served as control. The subsequent rate coefficients obtained after the addition of caffeine were computed for each 10 minute collection interval as the average of the data from six experimental runs. The units for outflux were converted from micromole/(gram minute) to micromicromole/(cm.^{2} sec.) so as to make comparison with influx easier.

From Table II the average outflux before the addition of caffeine is seen to be 0.082 μμmole/cm.^{2} sec., as compared to the figure of 0.096 μμmole/cm.^{2} for influx. The addition of caffeine raises the outflux, during the first 10 minutes to 0.132 μμmole/cm.^{2} sec., and by 20 minutes to a sustained level of approximately 0.229 μμmole/cm.^{2} sec. In Table I, the Ca influx in 0.005 M caffeine after 5 or 10 minutes' equilibration is seen to be 0.255 μμmole/cm.^{2} sec., so that outflux reaches approximately the same rate after 10 minutes' equilibration in the presence of caffeine.

The time lag for calcium outflux to reach maximum due to the filling up of the extracellular space can be calculated according to the mathematical analysis by Shanes (10). The half-time for emptying the extracellular space is 3 minutes (14), which corresponds to a time constant of 4.3 minutes. The value used for P_2, the penetration coefficient (the inverse of the time constant) which roughly describes the kinetics of filling or emptying the extracellular space, is 0.23 min.$^{-1}$. The average penetration coefficient (P_1) for the muscle membrane when increased threefold is 0.012 min.$^{-1}$. The calculated time for calcium to reach a maximum concentration (*tm*) in the extracellular space when outflux is increased threefold can be predicted to be 11 minutes.

Effect of Caffeine on Ca^{45} Outflux in Zero Ca and Zero Ca + 0.004 M EDTA

Paired sartorius muscles were soaked as usual for 4 hours in Ca^{45} Ringer's solution prior to washout. Both muscles of each pair were then washed in zero Ca Ringer's solution for 100 minutes; then 0.004 M EDTA was added and washout continued for another 80 minutes, when 0.005 M caffeine was added to one of the paired muscles and the washout continued for still another 70 minutes. A typical desaturation curve for one of three pairs of muscles

TABLE II

CALCIUM OUTFLUX IN FROG SARTORIUS MUSCLE

Row	No. of Experiments	Conditions	Outflux μμmols/cm² sec.*	Outflux during 10 min. collection intervals following addition of 0.005 M caffeine (μμmols/cm² sec.*) min. 10	20	30	40	50	60
A	6	Ringer's + 2 mg. per cent cocaine	0.082 ±0.009	0.132 ±0.015	0.266 ±0.037	0.266 ±0.026	0.237 ±0.031	0.218 ±0.010	
B‡	6	Ringer's + 14 mg. per cent cocaine	0.096 ±0.025	0.167§ ±0.026	0.190 ±0.017	0.180 ±0.014	0.142‖ ±0.011	0.127 ±0.017	0.131‖ ±0.010
C‡	6	0 Ca Ringer's + 14 mg. per cent cocaine + 0.004 M EDTA	0.123 ±0.018	0.337§ ±0.119	0.230 ±0.036	0.166 ±0.013	0.157‖ ±0.011	0.142 ±0.026	0.150‖ ±0.009

* Each figure the average of six experiments.

‡ Paired muscles were compared between B and C.

§ Caffeine present only during first 10 minute interval.

‖ Based on average of five experimental results instead of six.

is presented in Fig. 2. The addition of 0.004 M EDTA causes only a transient increase in the loss of Ca^{45}. The transient nature of the effect suggests that EDTA withdraws calcium from a surface component to the extracellular

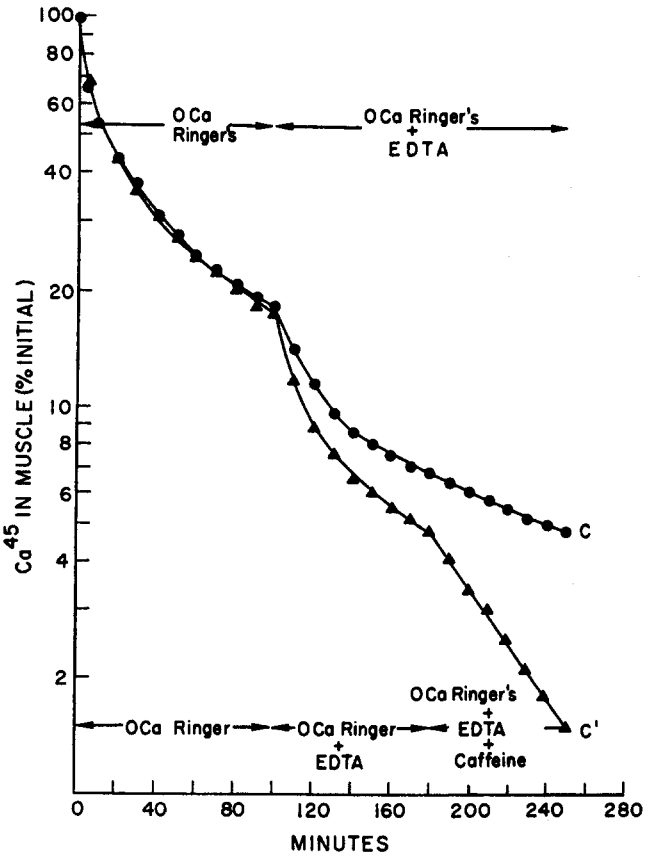

FIGURE 2. Time course of the decline of Ca^{45} content of paired muscles, C and C′, in zero calcium Ringer's solution, after prior exposure to Ringer's containing Ca^{45}. At 100 minutes 0.004 M EDTA is added to the medium of both C and C′, resulting in a release of Ca^{45} which tapers off after 140 minutes. At 180 minutes 0.005 M caffeine is added to zero calcium Ringer's plus 0.004 M EDTA bathing muscle C′, causing a sustained increase in Ca^{45} outflux.

phase, from where it diffuses out in the same manner as the initial fast component. Thus, EDTA is apparently capable of removing only a fraction of the calcium present in the muscle, a result in agreement with the findings of Gilbert and Fenn (5); moreover, the fraction involved appears to be a superficial component. Preliminary observations show that addition of calcium to the medium after EDTA fails to cause the usual transitory increase in Ca^{45} escape. When caffeine is added to the muscle after the EDTA, a marked

and maintained increase in Ca^{45} outflux occurs. The sustained effect by caffeine shows that it acts on the calcium remaining in the muscle after washout in EDTA.

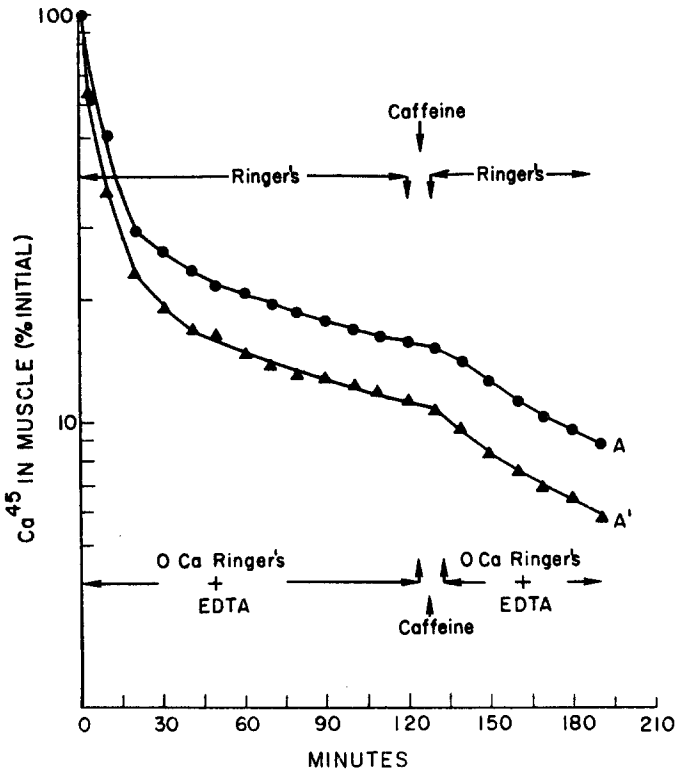

FIGURE 3. Time course of the decline of Ca^{45} content of paired muscles, A and A′, after prior exposure to Ringer's solution containing Ca^{45}. The Ca^{45} of muscle A is washed out in Ringer's solution containing 1 mM Ca, while that of muscle A′ is washed out in zero calcium Ringer's plus 4 mM EDTA. After 130 minute washout 5 mM caffeine is added to the solutions bathing both A and A′.

Effect of Brief Exposure to Caffeine on Ca^{45} Outflux in Ringer's Solution and in Zero Ca + 0.004 M EDTA Solution

After the usual exposure to Ca^{45} Ringer's solution, one muscle of each of six pairs was washed in Ringer's solution for 120 minutes, then treated for 10 minutes with 0.005 M caffeine, and finally returned to Ringer's solution for another 60 minutes. The second muscle of each pair was washed in zero Ca Ringer + 0.004 M EDTA for 190 minutes, except for exposure during the 120 to 130 minute interval to 0.005 M caffeine. All solutions in this series of experiments contained 14 mg. per cent cocaine. Representative desaturation curves are shown in Fig. 3.

The figures, and rows B and C in Table II, show that addition of caffeine

for a single 10 minute period increases Ca^{45} outflux from the muscle in Ringer's solution as well as from the muscle in zero Ca Ringer + 0.004 M EDTA; the increase is not sustained as well after removal of caffeine as when caffeine remains present.

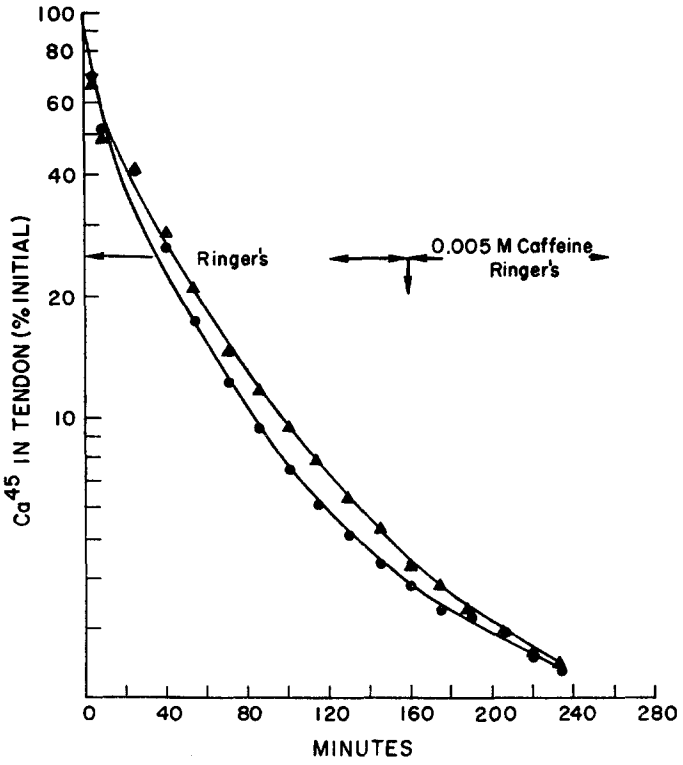

FIGURE 4. Time course of the decline of Ca^{45} content of paired tendons in Ringer's solution containing 1 mM calcium. The curve symbolized by a triangle represents a tendon that has been previously soaked in Ca^{45} Ringer's solution with 0.005 M caffeine for 4 hours. The curve symbolized by a solid circle represents a tendon that has been previously soaked in Ca^{45} Ringer's for 4 hours. At 160 minutes 0.005 M caffeine is added to the Ringer's solution. The two curves show no significant effect of caffeine on the decline of Ca^{45} from tendon.

Experiments on frog Achilles tendon were performed to determine whether caffeine affects the calcium of the interstitial connective tissue. Tendons soaked for 4 hours in Ca^{45} Ringer's solution with 0.005 M caffeine present did not take up any more Ca^{45} than their controls. Moreover, as may be seen in Fig. 4, caffeine has no effect on Ca^{45} emergence from these tissues.

DISCUSSION

The present findings show that 0.005 M caffeine increases both the calcium influx and outflux of muscle fibers. The increased influx of calcium occurs

equally well in potassium-depolarized muscle fibers, suggesting that calcium is being transferred in an uncharged form (*e.g.* as part of an ion pair (12)). Caffeine has been demonstrated to reduce the transverse membrane resistance slightly (1). If one estimates the contribution of the resting flux of calcium to the conductance of the membrane by use of the constant field relations (11), it is found to be only 0.5 μmho/cm.^{2} compared to recent measurements of the conductance of potassium, chloride, and sodium (respectively 100, 200, and 1 μmho/cm.^{2} (7, 8)). At best, trebling of calcium flux by caffeine would cause a resistance fall of the order of 0.3 per cent, compared to the 10 per cent actually found by Axelsson and Thesleff (1). The calcium fluxes therefore probably do not underlie the resistance change with caffeine.

The concentrations of caffeine used in these experiments did not produce a spontaneous contracture, but the effect on the contractile mechanism could be demonstrated by addition of 0.08 M KCl to the caffeine Ringer's solution. The KCl-induced contracture was prolonged by the presence of caffeine and could be reversed by washing away the caffeine Ringer's solution. KCl depolarization causes a large transient increase in calcium influx in the muscle fiber, presumably triggering off the contractile mechanism, which then can be maintained in a contracted state in the presence of caffeine. The threefold increase in calcium influx, which occurs in the presence of caffeine, does not seem to underlie the caffeine effect on the contractile mechanism, for an increase of this magnitude without change in outflux can be brought about by trebling the external calcium concentration. Furthermore, prevention of calcium influx by removal of external calcium does not prevent a caffeine contracture (1, 4). It is more likely that the increased calcium outflux brought about by caffeine reflects the process whereby caffeine affects the contractile mechanism. In the absence of extracellular calcium, caffeine still increases calcium outflux. If the proposal that the intracellular level of ionized calcium initiates the contractile response (2, 13) is accepted, then it is tempting to propose that increased calcium outflux brought about by caffeine reflects an increased intracellular level of ionized calcium. Caffeine can perhaps reduce the binding constant of sites for calcium in the membrane or perhaps in the sarcoplasmic reticulum and hence bring about an increase in the ionized calcium level, which would be reflected by the increased calcium outflux observed in the absence of extracellular calcium, and the increased outflux and influx in the presence of calcium, influx representing an exchange of extracellular calcium for intracellular calcium. The increased calcium influx and outflux could also be a consequence of change in permeability to calcium; *i.e.*, membrane changes resulting in a greater mobility of calcium through the membrane. The slight change in membrane resistance produced by caffeine, and lack of a membrane depolarization suggest that caffeine does not cause a general increase in membrane perme-

ability. Decrease of calcium binding by intracellular and membrane sites, rather than a permeability increase, seems more attractive as the basis for increased calcium efflux, since EDTA, which would be expected to increase permeability by removal of membrane calcium, causes only a transitory increased loss of Ca^{45}.

Caffeine and potassium depolarization affect different sites concerned with calcium movement. Increased potassium depolarization causes a marked transient increase in calcium influx and outflux (15) which return to a low level upon sustained depolarization, and KCl contracture is dependent upon extracellular calcium (4). Caffeine causes a sustained increase in calcium influx and outflux, and the caffeine contracture does not depend upon the presence of extracellular calcium. The site of caffeine action with regard to the contractile mechanism is on the membrane, for Axelsson and Thesleff (1) have demonstrated that only caffeine applied externally results in contracture. Caffeine applied by injection to the muscle interior is without effect. The two different types of contractures indicate that there are at least two ways in which contractures can be brought about; one by a membrane depolarization in which case calcium influx is necessary; the other, perhaps, by interfering with membrane or sarcoplasmic reticulum binding sites for calcium, so that the intracellular calcium ion level is increased.

In summary, then, it may be stated that the effects of caffeine on calcium movement in muscle are not inconsistent with previous evidence for the critical role of the divalent ion in contractile phenomena, in contradiction to conclusions reached from less direct observations with caffeine. Moreover, the negligible membrane potential changes induced by caffeine (1), in sharp contrast to the effects on calcium flux observed in the present study, provide further evidence in keeping with earlier observations (4) that the bioelectrical changes are secondary to calcium in the link between excitation and contraction. These findings do not rule out the possibility that other components associated with calcium movement have a more primary role in the initiation and strength of contracture, but neither do they demand it. Under the circumstances, the simpler possibility of a direct involvement of calcium in contracture and contraction seems to remain preferable.

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