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CALCIUM UPTAKE AND FORCE DEVELOPMENT BY SKINNED MUSCLE FIBRES IN EGTA BUFFERED SOLUTIONS

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SUMMARY

1. The uptake of ^{45}Ca and the development of force by segments of skinned muscle fibres were measured in Ca solutions buffered with EGTA. When the Ca ion concentration in these solutions was above the contraction threshold, Ca was accumulated by the sarcoplasmic reticulum during the delay phase before force developed. The uptake rate increased, and the length of the delay decreased, when the concentration of the calcium buffer was increased.

2. The maximum accumulation of Ca was 2–3 mM. Force developed, ending the delay phase, when the calcium uptake approached this maximum level.

3. The pattern of force development suggested that this process was often accompanied by a sudden release of accumulated Ca.

4. The relation between the kinetics of ^{45}Ca uptake and the interaction of calcium with EGTA and the sarcoplasmic reticulum is discussed. The data indicate that at the low concentrations necessary for relaxation the sarcoplasmic reticulum takes up Ca fast enough to account for the rate at which force falls when intact muscle fibres relax.

INTRODUCTION

The ability to buffer Ca ion concentration in the micromolar range with EGTA (ethyleneglycol-bis-(aminoethylether)-N-N′-tetra-acetic acid) has made it possible to study the contractile properties of skinned muscle fibres in solutions of known chemical composition (Hellam & Podolsky, 1969; Ford & Podolsky, 1970; Podolsky & Teichholz, 1970; Gordon, Godt & Woodbury, 1970; Endo, Tanaka & Ogawa, 1970). Steady force is a function of the pCa of the bathing solution, but the rate of force development

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is generally much slower than that expected from simple diffusion of a substance from the bathing solution into the fibre. Hellam & Podolsky (1969) suggested that the delay in force development is due to slow equilibration of the EGTA buffer system during Ca uptake by the sarcoplasmic reticulum. Since this explanation sets limits upon the rate constants for the Ca uptake processes in the physiological concentration range, it seemed worth while to study the delay mechanism in greater detail.

The present report describes experiments in which the uptake of Ca during the delay phase was measured directly with ^{45}Ca. The influence of the total EGTA concentration on the length of the delay phase in solutions at a given pCa was also examined. The time course of force development indicated that the end of the delay phase was often associated with release of Ca from a source within the fibre volume, presumably the sarcoplasmic reticulum. Direct evidence for this release process is put forward in the second paper of this series.

A preliminary account of this work has already been reported briefly (Ford & Podolsky, 1968).

METHODS

Fibre preparation

The technique of Hellam & Podolsky (1969) was changed to permit the use of a number of segments from the same fibre. The fibres were kept cold throughout the dissection procedure to minimize deterioration. Immediately after removal from the frog, the semitendinosus muscle was suspended in a cold (4–10° C) 200 mM sucrose solution containing 8 mM-NaCl and 3·3 mM-$NaH_2PO_4 + Na_2HPO_4$ at pH 7·2–7·4; this solution was used in place of the usual Ringer solution to reduce the diffusion of Ca and Cl ions into the cut ends of the fibre during dissection. Whole muscles were stored in this solution at 4° C for up to 3 hr before bundles of fibres were removed. The bundle was rinsed for 15 sec in a bathing solution (see section below) containing 1·0 mM-EGTA, blotted dry, and placed on a slide in a dish containing silicone oil that had been kept cold in an ice chest. One of the larger fibres in the bundle was isolated along most of its length and divided into 4–12 segments. The segments were transferred to individual slides, which were then stored in silicone oil at 0° C. Immediately before use, each segment was skinned and transferred, with its slide, to silicone fluid at room temperature, where it was mounted at rest length (sarcomere length 2·0–2·1 μ) in the force measuring apparatus. The apparent diameter of some fibres was recorded at this stage by photographing the preparation in the plane of the fibre axis using a long working-distance objective (Leitz UMK 50).

Force transducer and solution changer

The method of Hellam & Podolsky (1969) was modified by reducing the silicone fluid over the bathing solutions to a thin layer, intended only to retard evaporation. As a result, the fibre was moved through an air:silicone:water interface to change solutions. This modification reduced the time required for solution changes to 1–2 sec. The time of the change was indicated to the nearest half second by a small mark on the force record.

Bathing solutions

Unless otherwise indicated, the experimental solutions were prepared by mixing three stock solutions in various proportions: (*A*) 120 mM-K propionate, 1 mM-$MgCl_2$, 5 mM-Na_2ATP, 2 mM procaine, and 10 mM imidazole at pH 7·00 (± 0·02); (*B*) *A* + 10 mM-CaEGTA; (*C*) *A* + 10 mM-EGTA. Buffered calcium solutions were made by mixing *B* and *C* in the appropriate ratio; this mixture was then diluted with *A* to give the desired concentration of *total* EGTA, $[EGTA]_0$ = [EGTA] + [CaEGTA]. The pH of such solutions was found to be within 0·02 units of pH 7·00 without further adjustment. The stability constant used to calculate the pCa was 6·7. A few runs were made with 5 mM-ATP replaced by an ATP regenerating system consisting of 1 mM-ATP, 4 mM creatine phosphate, and creatine phosphokinase 0·2 mg/ml. Procaine was omitted from some of the solutions when the time course of force development by fibre segments was studied. The experiments were done at room temperature (21 ± 1·5° C).

K propionate was prepared by neutralizing propionic acid with KOH. Inorganic salts, propionic acid, and sucrose were the reagent grade supplied by the Fisher Scientific Company. Imidazole was obtained from Eastman Organic Chemicals, procaine hydrochloride from Z. D. Gilman Company as the 30–80 mesh USP grade powder, EGTA from Geigy Industrial Chemicals, and Na_2ATP from Sigma Chemical Company as the 99–100 % pure, low calcium content reagent.

^{45}Ca was obtained from the New England Nuclear Corporation with a specific activity of approximately 15 c/g in solutions containing 10 mM-Ca. These solutions were not re-assayed for Ca concentration but were diluted 1:9 with an unlabelled 10 mM Ca solution to reduce any error in the supplier's assay by tenfold.

Radioactive calcium uptake

The uptake by fibre segments in different experimental solutions was expressed as a percentage of the uptake by one or two segments of the same fibre immersed for 30 sec in a reference solution containing 0·375 mM-Ca and 0·5 mM total EGTA (pCa 6·2). The specific activity of the Ca in the experimental solutions was generally the same as that in the reference solution; when this was not the case, the percentage uptake was corrected according to the ratio of the two specific activities. The concentration of Ca taken up was determined from volume measurements on some of these reference segments, enabling the uptake of Ca to be expressed in absolute, as well as in relative, amounts.

After skinning, the fibre segments were equilibrated with a 0·1 mM-EGTA bathing solution for 10 sec before being transferred to solutions containing ^{45}Ca. Following the exposure to ^{45}Ca, the segments were rinsed in a 0·1 mM-EGTA bathing solution for 10 sec before being dried and assayed for radioactivity.

Dehydration by ethanol does not remove bound Ca from the fibres (L. Costantin & R. J. Podolsky, unpublished observation). All fibres were therefore dehydrated in three 20-sec exposures to absolute ethanol to facilitate subsequent air drying by gentle aspiration. The dried fibres were removed from the transducer forceps and placed on planchets containing double-sided Scotch tape. Portions of the fibres that had been in the forceps were cut off and the remaining lengths measured with a reticle in the eyepiece of a Zeiss dissecting microscope set at a magnification of 40 ×. Radioactivity in the fibres was measured with a Beckman Lowbeta II planchet counter having a background of less than 0·5 cpm. The segments were counted for at least 2000 counts. Radioactivity content was expressed first as cpm/mm fibre length, and subsequently the Ca concentration was determined as a percentage of that in the reference segments.

The sequence of exposure of segments to experimental conditions was ordered to eliminate any systematic error that might have resulted from storage of the segments. This was generally done by testing a set of conditions in sequence. and then reversing the sequence, using segments from the same fibre.

Control experiments

Reproducibility of Ca uptake. Two segments of a fibre, usually the second and seventh to be tested, were used as a reference and allowed to accumulate Ca for 30 sec in the reference solution. These segments from 100 fibres were used to examine the reproducibility of the Ca uptake method. The radioactivity content of the seventh

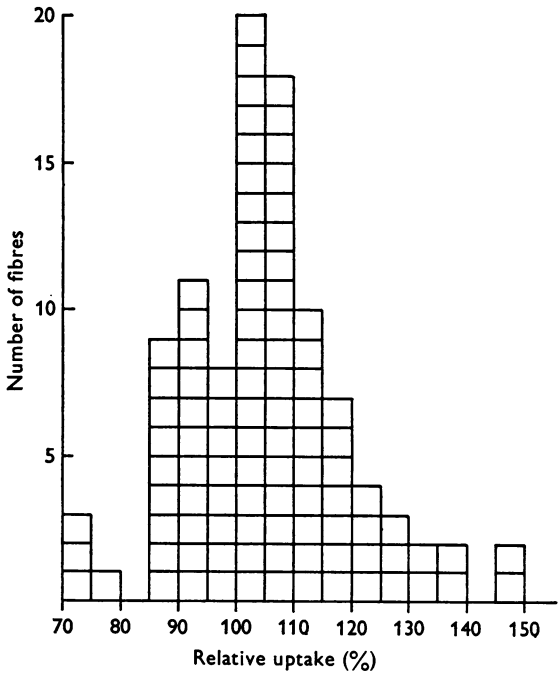

Fig. 1. Comparison of the ^{45}Ca uptake per unit length of two segments from the same fibre. Each fibre was divided into at least seven segments. The second and seventh segments were immersed for 30 sec in a reference solution at pCa 6·2 containing 0·375 mM-^{45}Ca and 0·5 mM total EGTA in addition to the standard reagents. The graph shows the frequency distribution for the radioactivity content per unit length of the seventh segment expressed as a percentage of that of the second segment.

segment was expressed as a percentage of the second, and the number in each 5% interval is plotted in Fig. 1. The average value for the distribution is 105%, which indicates that storing the segments in cold silicone fluid did not impair their ability to accumulate calcium. The S.D. of 14% is a measure of the reproducibility of the method.

Effect of storing whole muscles. Muscles were frequently stored in the cold sucrose solution for several hours before individual fibres were removed. The effect of storage was examined by comparing Ca uptake by a fibre that had been stored with that of a

fibre of nearly the same diameter from the muscle before storage. The radioactivity content of both reference segments was averaged for each of two fibres and the uptake by the segments of the second fibre expressed as a percentage of that by the first. The average value of the distribution for sixteen muscles was 110 % (S.D. 19 %), which indicates that storing the muscle in the cold sucrose solution had no significant effect on the ability of the fibres to accumulate Ca.

Ca efflux in EGTA solutions. This was measured by immersing segments of a fibre in the reference solution for 30 sec and varying the duration and concentration of the EGTA rinse. The control for these experiments was the 10-sec rinse in 0·1 mM-EGTA

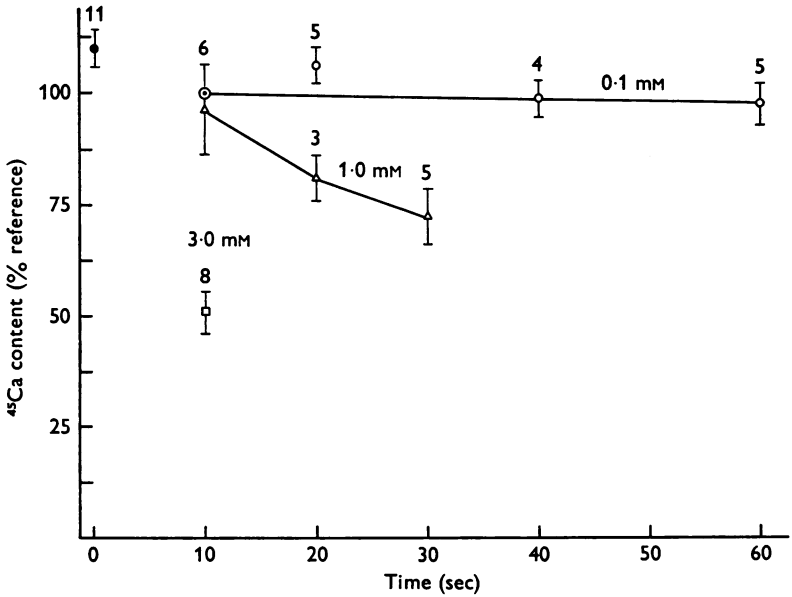

Fig. 2. Ca efflux from skinned muscle fibres in EGTA solutions. Fibre segments that had accumulated ^{45}Ca in the reference solution for 30 sec were rinsed in 0·1 mM (○), 1·0 mM (△) or 3 mM (□) EGTA solutions for the times given on the abscissa. The ^{45}Ca content, relative to that present after a 10 sec 0·1 mM EGTA rise (⊙), is the ordinate. In addition to EGTA, the rinse solutions contained 120 mM-K propionate, 5 mM-ATP, 1 mM-$MgCl_2$, 10 mM imidazole, 2 mM procaine at pH 7·0, 21° C. Segments were also counted with no rinse (●). Vertical bars give S.E. of the mean for the indicated number of segments. A small amount of Ca, indicated by the difference between the ordinates for points ● and ⊙, is quickly removed by the 0·1 mM-EGTA rinse. The Ca that remains bound to the fibre in 0·1 mM-EGTA is removed slowly by 1·0 mM-EGTA and more quickly by 3·0 mM-EGTA.

used for the uptake experiments. The Ca content of the experimental segments was compared with that of one or two segments of the same fibre given this standard rinse. In addition, eleven segments were not rinsed in EGTA but were transferred directly to alcohol from the ^{45}Ca solution. As shown in Fig. 2, the mean radioactivity content of these eleven segments was about 10 % higher than that of their reference segments; this is probably due to ^{45}CaEGTA in the myofilament space that was removed by the 10-sec rinse in 0·1 mM-EGTA. Fig. 2 also indicates that the fibres

retain the Ca present after the standard rinse when immersed in 0·1 mM-EGTA, but lose it in higher concentrations of EGTA. Thus, in the uptake experiments, the 10 sec rinse in the 0·1 mM-EGTA bathing solution appears to be adequate to wash out the free Ca without removing a significant amount of bound Ca from the fibres. A further reason for using a low concentration of EGTA was that some of the fibres contracted spontaneously after being loaded with Ca, apparently from the release of stored Ca, and it was considered important not to prevent these contractions with EGTA, as this might mask some Ca loss.

Spontaneous contractions and the use of procaine. A fibre that had accumulated a large amount of Ca would occasionally contract uniformly along its length when moved to a new solution. The contractions occurred in solutions without Ca (Fig. 3*a*), and could be interrupted by high concentrations of EGTA (Fig. 3*b*), which

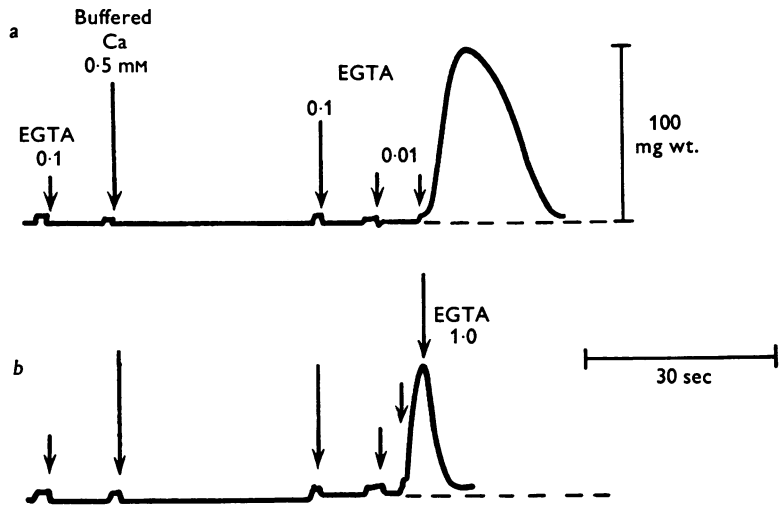

Fig. 3. Spontaneous contractions of skinned muscle fibres. Arrows mark immersion in solutions containing Ca buffered to pCa 6·2 with $[EGTA]_0$ = 0·5 mM, or EGTA in the millimolar concentrations specified. A fibre that had accumulated Ca from a buffered solution occasionally produced a contraction 'spike' when moved through the surface of a solution containing a low concentration of EGTA (*a*, *b*). This spike could be interrupted by increasing the EGTA concentration (*b*). The incidence of these 'spontaneous contractions' was increased when procaine was omitted from bathing solutions, as it was in these examples.

indicates that they were due to Ca in the myofilament space that had come from a source within the fibre volume. The response was seen most often in preparations from winter and/or poorly nourished frogs.

The frequency of the response was decreased significantly, although not eliminated, with 2 mM procaine in the solutions. Because these contractions appeared to be associated with the release of stored Ca, no segment which gave this response was taken for ^{45}Ca determination, and to reduce the number of wasted segments, 2 mM procaine was included in all experiments involving radioactive Ca measurements.

Effect of procaine on ^{45}Ca uptake. The effect of procaine on the rate of Ca accumulation was determined by measuring the uptake from a solution with and without

procaine, as shown in Fig. 4. The dashed line (taken from Fig. 6) represents the Ca uptake from the reference solution which contained 2 mM procaine. The data points were obtained with a solution which was the same as the reference except that procaine was omitted. The rate of Ca accumulation does not appear to be significantly affected by procaine.

Effect of procaine on force development. The influence of procaine on the contractile elements and the Ca buffer system was tested by measuring the steady force produced by fibres in the presence and absence of procaine. Skinned segments were placed in a solution at pCa 6·5 without procaine, in which they produced nearly half

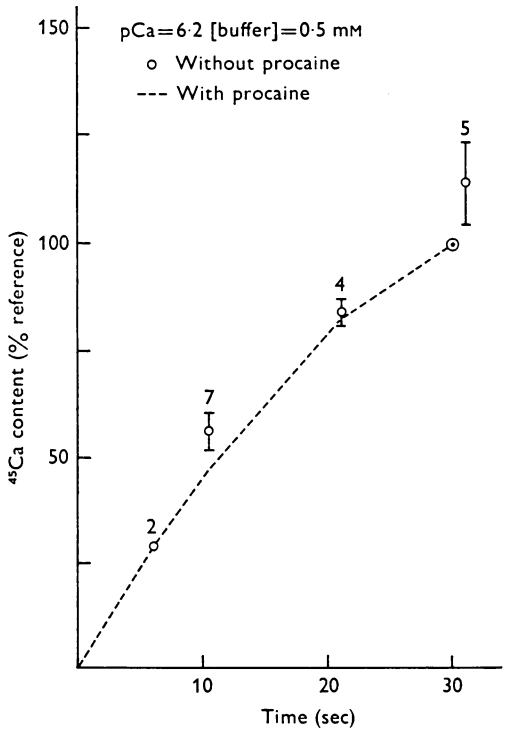

Fig. 4. Effect of 2mM procaine on Ca uptake by skinned muscle fibres. The uptake of ^{45}Ca from the reference solution, given by the dashed line, is taken from Fig. 6; ⊙ indicates the average uptake by the reference segment which defines 100 % on the ordinate. The vertical bars for the solutions without procaine (○) show the S.E. of the mean for the indicated number of fibre segments.

maximum force, and were then transferred to an identical solution to which 2 mM procaine had been added. The absence of a force change indicated that procaine did not affect either the force generating mechanism of the fibre or the pCa of the bathing solution (or that both factors had been changed by exactly compensating amounts). The effect of procaine was further tested by varying the total EGTA concentration at a given pCa, keeping the procaine concentration at 2 mM. Fibres were equilibrated with solutions nominally at pCa 6·5 with $[EGTA]_0$ ranging from 0·5 to

4·0 mM. The force remained constant throughout, which indicated that the pCa of the solutions did not change significantly at different concentrations of the Ca buffer and therefore that procaine did not interact with the buffer system.

RESULTS

Time course of force development

The threshold for force development in the present solutions was about pCa 6·7, and maximum steady force was produced at pCa 6·0. The delay in the onset of force at pCa 6·2 is shown in Fig. 5; this pCa was selected for study because it gave nearly maximum force in the steady state but did not mask force transients.

When the total concentration of EGTA in the pCa 6·2 solution was 2·0 mM, the duration of the delay was relatively short, as in tracing 5*a*. When the buffer concentration was lowered, the average length of the delay increased (Fig. 5*b*, *c* and *d*). Some representative values from fibres in solutions maintained at pCa 6·2 with different concentrations of EGTA buffer are given in Table 1.

The contractions which ended the delay at the lower concentrations of total EGTA showed striking variations. The slow, monotonic rise of force in Fig. 5*b* was seen only infrequently. More often, the onset of force was sudden, as in Fig. 5*c*. Frequently there was an overshoot of force above the steady level, and occasionally a 'spike' was superimposed on a slowly rising contraction, as in Fig. 5*d*. Rarely, a steady force was not established and only repetitive spikes occurred, as in Fig. 5*e*. These last three tracings suggest that Ca was liberated from sites within the fibres during the contractions. When observed with the dissecting microscope, these contractions appeared to be uniform along the length of the preparation.

The overshoot was much reduced, or absent, when solutions containing higher concentrations of total EGTA were used. Thus, the pattern of force development seen by Hellam & Podolsky (1969), using solutions containing Ca buffered with 3 mM total EGTA, was generally like that shown in Fig. 5*a* and *b*.

Ca uptake during the delay phase

pCa 6·2. The uptake of ^{45}Ca by a fibre in a solution maintained at pCa 6·2 by various concentrations of $[EGTA]_0$ is shown in Fig. 6. Ca accumulation began as soon as the fibre was placed in the ^{45}Ca solution, which shows that the delays in force development were not due to exclusion of Ca from the fibre.

The threshold for force development in these solutions was close to pCa 6·7. Since the Ca ion concentration in the myofilament space of the fibre must be below this level during the delay phase, the Ca uptakes in Fig. 6

show the ability of the internal membrane system to take up Ca from concentrations below the contraction threshold.

Both the initial (Table 2) and the average uptake rate increased when the EGTA concentration was increased. On the other hand, the average delay became shorter when the EGTA concentration was increased (Table1).

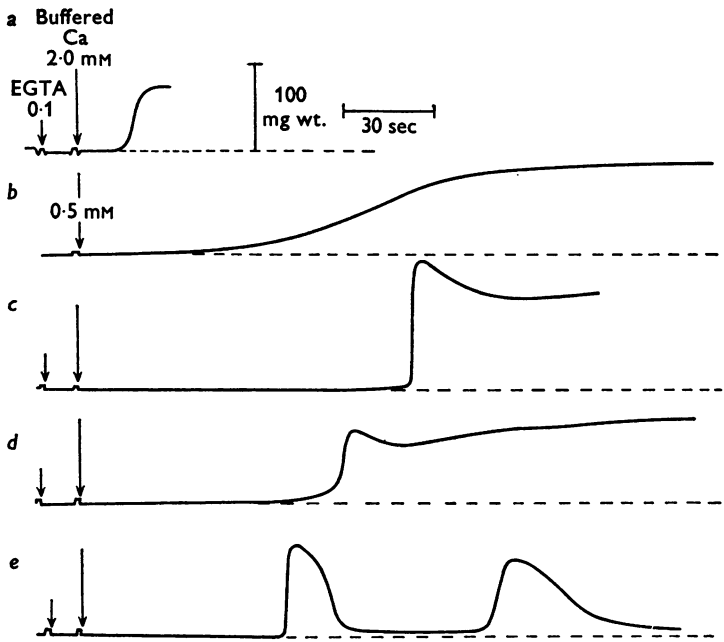

Fig. 5. Force development by skinned fibres in buffered Ca solutions. Fibre segments were equilibrated in a solution containing 0·1 mM-EGTA for about 10 sec and then transferred to Ca solutions buffered to pCa 6·2 with a total EGTA concentration of 2·0 mM (*a*) or 0·5 mM (*b–e*). The delay in the 0·5 mM solution was longer than that in the 2·0 mM solution and the pattern of force development was more variable. Force sometimes developed slowly and monotonically (*b*), but more often developed rapidly with an overshoot (*c*, *d*). In a few cases a steady force was not established and repetitive spikes were seen (*e*). Procaine was omitted from the solutions in *b*, *c*, and *e*; an ATP regenerating system was used in *e*.

TABLE 1. Delay in force development by skinned fibres in EGTA buffered solutions at pCa 6·2. The duration of the delay was measured from the time of immersion in the calcium buffer to the time when force reached about 10 % of its steady level

$[EGTA]_o$ (mM)	Number of fibres	Mean delay (sec)	Range (sec)
2·0	18	16	5–40
0·5	17	99	55–180
0·1	4	131	95–195

These observations suggest that one of the factors that determined the end of the delay phase was the accumulation of a fixed amount of Ca by the internal membranes.

Maximum measurable uptake rate. This was taken as the uptake of fibres in a solution adjusted to pCa 6·0 with $[EGTA]_0$ = 4·5 mM. The average delay in this solution was only a few seconds, but fibres were selected in which the delay was long enough (at least 4 sec) to make an uptake rate measurement. The average uptake per sec for these fibres was 30 % of the reference uptake (nine preparations in June, 1968, uptake time 4–6 sec, standard deviation 3 % reference.sec^{-1}). As discussed below, this corresponds to Ca uptake at a rate of 0·27 mM.sec^{-1}.

TABLE 2. Initial rate of Ca uptake, v, by skinned fibres in EGTA buffered ^{45}Ca solutions. $[EGTA]_0$, [CaEGTA], and [EGTA] denote the concentration of total EGTA, the Ca complex, and the uncomplexed species, respectively, in the bathing solution

	pCa	$[EGTA]_0$ (mM)	[Ca EGTA] (mM)	[EGTA] (mM)	v (% ref. sec^{-1})	v (mM.sec^{-1})	v/[Ca EGTA] (sec^{-1})
1	6·2	0·1	0·075	0·025	0·83	0·0075	0·10
2	6·2	0·5	0·375	0·125	4·4	0·040	0·11
3	6·2	2·0	1·5	0·5	12	0·11	0·07
4	6·0	4·5	3·75	0·75	30	0·27	0·07
5	7·0	2·25	0·75	1·5	5·3	0·05	0·07

Maximum accumulation of Ca

The segments used to obtain the data in Fig. 6 remained relaxed throughout both the period of uptake and the EGTA rinse. The uptake of Ca by relaxed fibres could not be studied in detail for periods much longer than those shown because the segments would then often contract spontaneously when moved into the rinse solution. Fibres that had been left in the 0·5 and 2·0 mM $[EGTA]_0$ solutions at pCa 6·2 for sufficient periods to produce steady contractions were found to accumulate 2–3 times the Ca taken up by the reference segments (mean 2·2, S.D. 0·5, 23 segments).

The curves in Fig. 6 for 0·1, 0·5 and 2·0 mM total EGTA indicate that the fibres would have taken about 265, 85, and 30 sec, respectively, to accumulate 2·2 times the reference uptake. For the 0·5 and 2·0 mM solutions, these times are within the range given for the delays in force development in Table 1, which suggests that contraction in these solutions began when Ca accumulation approached the maximum; in the 0·1 mM solution, the delay apparently ended before the capacity of the internal membrane system for calcium had been reached.

Ca uptake at pCa 7

Since the threshold for force development in the present solutions was about pCa 6·7, fibres immersed in a bathing solution at pCa 7·0 remained relaxed indefinitely (5–10 min). Fig. 7 shows the uptake of ^{45}Ca from such a solution. The uptake began immediately and then continued at a slower rate until the fibres had accumulated close to three times the Ca bound by the reference segments. The final level was close to that found at pCa 6·2 after steady force had developed.

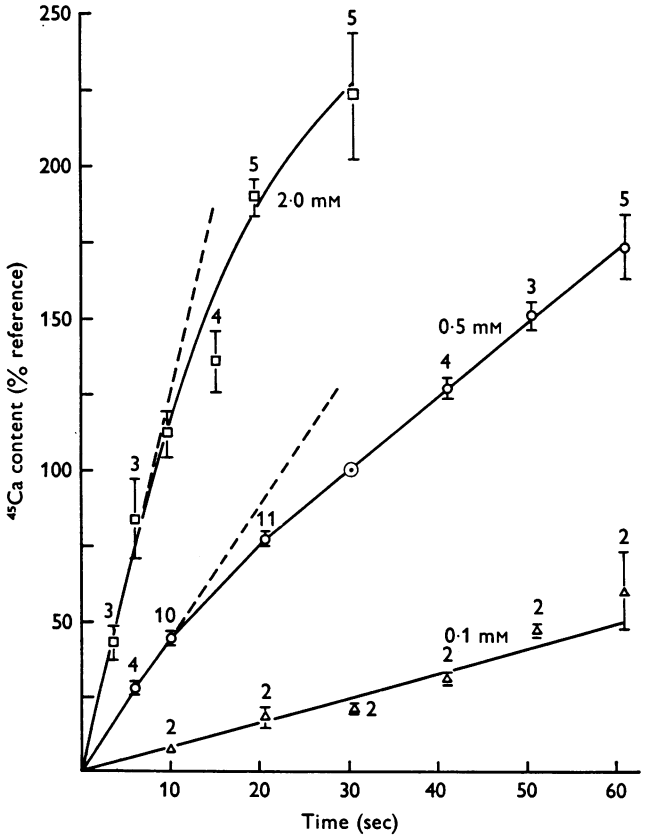

Fig. 6. Ca uptake from solutions at pCa 6·2. Fibre segments were immersed for various times in solutions containing ^{45}Ca buffered with 0·1 mM (△), 0·5 mM (○), 2·0 mM (□) total EGTA to pCa 6·2 and the radioactive Ca content of the fibres was assayed. At least one, and usually two segments of a fibre were immersed in the reference solution for 30 sec, (⊙). The Ca content per unit length of other segments from the fibre was expressed as a percentage of that in the reference segments and plotted as a function of the time of immersion. The curves were fitted by eye; the estimate of initial uptake rate is shown by a dashed line. Vertical bars give the S.E. of the mean for the indicated number of segments.

Absolute uptake of Ca by the reference segments

The concentration of Ca taken up by each of twelve reference segments was calculated from (*a*) the radioactivity of the segments, (*b*) the specific activity of the ^{45}Ca in the reference solution, (*c*) the length of the segments, and (*d*) the apparent diameter of the preparation in silicone fluid. The average diameter, estimated from photomicrographs, was 96 μ (standard

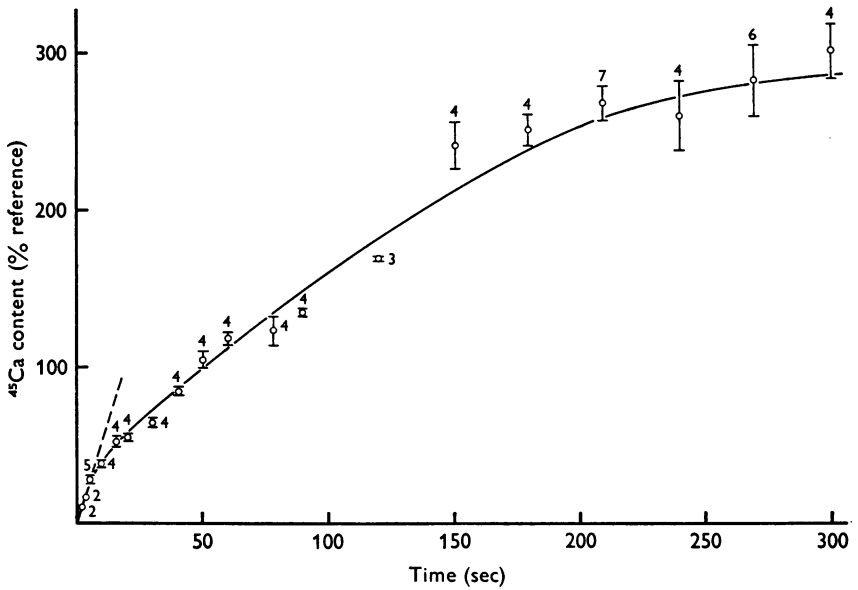

Fig. 7. Ca uptake from a solution at pCa 7·0. ^{45}Ca uptake from a solution buffered to pCa 7·0 with 2·25 mM total EGTA was measured as in Fig. 6. Fibres did not contract at this pCa, and the uptake was followed until it reached a plateau. Vertical bars give the S.E. of the mean for the indicated number of preparations.

deviation 14μ). The average Ca accumulation was found to be 0·9 mM (S.D. 0·2 mM). Thus the amount of Ca (in m-mole/l. fibre vol.) taken up in the experimental segments can be found by multiplying the percentage of reference uptake by $0{\cdot}9 \times 10^{-2}$.

Because the assumption of a round cross-section may lead to an overestimate of the fibre volume (Blinks, 1965), the absolute value for the uptake may be too small. This error is balanced, to some extent, by the fact that skinned fibres appear to lose water to silicone fluid, which reduces the fibre diameter by about 20% (S. Hatchett & R. J. Podolsky, unpublished observation). Neither of these factors introduces an error in the estimate of the original fibre volume of more than 50%, and this was, therefore, taken as the error limit of the absolute measurement.

DISCUSSION

General remarks

The main findings of the present study are (*a*) in bathing solutions buffered with EGTA to Ca ion levels above the contraction threshold, significant amounts of Ca are accumulated by the fibre during the delay phase; (*b*) the Ca uptake rate at a given pCa increases when the total EGTA concentration is increased; and (*c*) the duration of the delay phase is inversely related to the Ca uptake rate. As will be discussed below, these observations are consistent with the idea that the delay in force development is due to slow equilibration of the EGTA buffer system during Ca uptake by the sarcoplasmic reticulum (SR) (Hellam & Podolsky, 1969).

The amount of Ca that binds to the myofilaments when full force is produced is about 0·1 m-mole/l. fibre volume (Weber & Herz, 1963; Ebashi & Endo, 1968). The fact that many times this amount is taken up by the fibre before force develops shows that Ca exclusion from the fibre is not an important factor in the delay mechanism (however, see the discussion of diffusion below). It is also evidence that almost all the calcium taken up by the fibre during the delay phase is bound by the SR. Mitochondrial uptake is probably negligible since fibres from the semitendinosus muscle of the frog contain relatively few mitochondria and, more significantly, the affinity of the calcium transport system in mitochondria is much lower than that of reticulum (Weber, 1966).

Rate of Ca uptake during the delay phase

The rate of calcium uptake by the SR in the steady state is related to the reactions of the EGTA system by the equation

$$v = k_1[\mathrm{CaEGTA}]_i - k_2[\mathrm{Ca}]_i[\mathrm{EGTA}]_i, \tag{1}$$

where v is the uptake rate, and k_1 and k_2 are 0·4 sec^{-1} and 2×10^6 M^{-1} sec^{-1}, respectively (Hellam & Podolsky, 1969), and the subscript i denotes concentrations within the fibre volume under consideration.

For Ca ion concentrations below pCa 6, the rate of ATP hydrolysis by solubilized SR is proportional to the Ca ion concentration (Weber, 1971), which makes it reasonable to suppose that the initial rate of Ca uptake by the SR is also proportional to $[\mathrm{Ca}]_i$,

$$v = \xi[\mathrm{Ca}]_i, \tag{2}$$

where ξ is the appropriate rate constant. In this case a lower limit for the value of ξ can be taken as the ratio of the maximum uptake rate measured during the delay phase (Table 2, row 4) and $10^{-6.7}$ because the Ca ion level

within the fibre during this phase must be less than pCa 6·7, the contraction threshold for the conditions of the present study. This consideration puts the lower limit for ξ at $1{\cdot}4 \times 10^3$ sec^{-1}.

This argument gives a limiting rather than an absolute value for ξ because the Ca level inside the fibre during the delay phase is not known. The relatively short delay associated with conditions that gave the maximum uptake rate could indicate that the internal Ca ion concentration was close to the contraction threshold. However, technical constraints and diffusion limitations set the minimum time in which an uptake measurement can be made at about 4 sec. Since the SR takes up only 2–3 mM-Ca, the greatest initial uptake rate that can be measured is one in which a substantial fraction of this amount is accumulated in these 4 sec. The maximum rate found in the present study, about 1 mM Ca/4 sec, is of this magnitude. Therefore the short delay in that case probably reflects filling of the SR, and all that can be said about the concentration of ionized Ca inside the fibre before force developed is that it was *below* the contraction threshold.

Combination of eqns. (1) and (2) gives

$$v = \frac{k_1[\text{CaEGTA}]_i}{1 + k_2\,[\text{EGTA}]_i/\xi}. \tag{3}$$

The magnitudes of k_2 and ξ are such that for concentrations of free EGTA present in the bathing solutions during the uptake measurements (Table 2) the denominator on the right side of eqn. (3) is always between 1 and 3. Under these conditions the rate of calcium uptake would be determined primarily by the value of $k_1[\text{CaEGTA}]_i$, the rate of dissociation of the CaEGTA complex inside the fibre volume, although free EGTA at high concentrations would compete to a certain extent with the SR for Ca ions within the fibre. The data in the final column of Table 2 suggest that this is the case, as the uptake rate is roughly proportional to [CaEGTA], the concentration of CaEGTA in the bathing solution, over a fiftyfold range. The change in the ratio v/[CaEGTA] from 0·10 to 0·07 sec^{-1} when [EGTA] increases from 0·025 to 1·5 mM probably reflects the competitive effect of free EGTA within the fibre volume at the higher levels of this ion.

The intracellular CaEGTA concentration

According to eqn. (3), $[\text{CaEGTA}]_i = v/k_1$ when $[\text{EGTA}] \ll \xi/k_2$. Since the lower limit for ξ is $1{\cdot}4 \times 10^3$ sec^{-1} and $k_2 = 2 \times 10^6$ M^{-1} sec^{-1}, the inequality is satisfied when $[\text{EGTA}]_i \ll 0{\cdot}7$ mM, which is likely to be the case for the conditions specified in rows 1 and 2 of Table 2. The ratio of the uptake rates observed under these conditions and k_1 gives values for $[\text{CaEGTA}]_i$ that are about 4 times lower than the corresponding concentrations of CaEGTA in the bathing solution. This apparent decrease in concentration can be attributed to the concentration gradient associated with the diffusion of CaEGTA into the fibre and to the exclusion of this ion from part of the fibre volume.

The sarcoplasmic reticulum occupies 13 % of the fibre volume (Peachey, 1965); the myofilaments take up 10 % (if the diameters of the two types of myofilament are 50 and 110 Å (Huxley & Hanson, 1960)) to 25 % (if the diameters of the myofilaments are 100 and 200 Å (Elliott, Lowy & Worthington, 1963)) of the remaining volume. Therefore CaEGTA is probably excluded from 22–35 % of the fibre volume, and its concentration calculated on the basis of total fibre correspondingly reduced.

The influence of diffusion can be estimated by a series of approximations when the second term on the right of eqn. (1) is negligible, that is, when the free EGTA concentration inside the fibre is relatively low. In this case, all the Ca ions released by dissociation of the CaEGTA complex within the fibre are taken up by the SR and the Ca uptake rate is directly proportional to the concentration of CaEGTA. A similar problem has been worked out in the treatise by Crank (1957), which contains an expression for the influence of fibre diameter and diffusion coefficient on the uptake rate for a cylindrical fibre in a well stirred solution. From known values of ionic mobility in muscle cells (Kushmerick & Podolsky, 1969), the diffusion coefficient of CaEGTA is estimated on the basis of molecular weight to be $1{\cdot}6 \times 10^{-6}$ cm^2 sec^{-1}. Then the Ca uptake rate in a 100 μ diameter fibre with $k_1 = 0{\cdot}4$ sec^{-1} is calculated to be 0·57 times the rate that would be generated by CaEGTA uniformly distributed throughout the available fibre volume at the concentration in the bathing solution (eqn. (8.106); Crank, 1957).

To take into account the fact that the actual bathing solution was not stirred, the factor calculated in the above paragraph should be multiplied by a number close to 0·5. This estimate was made by comparing diffusion across a plane into a semi-infinite medium from (*a*) a stirred solution of concentration C_0 (eqn. (2.45); Crank, 1957), and (*b*) unstirred solution initially at concentration C_0 (eqn. (2.14); Crank, 1957). The value of the concentration at any distance inside the medium in case *a* is twice that for case *b*. Therefore the flux into the medium, which is proportional to the concentration gradient at the boundary plane, is twice as large when the bathing solution is stirred than when it is unstirred.

In summary, the average concentration of CaEGTA within the fibre would appear to be less than that in the bathing solution because (i) only 0·65–0·78 of the fibre volume is accessible to CaEGTA, (ii) finite diffusion times inside the fibre reduce the Ca uptake rate to 0·57 of that expected for CaEGTA in equilibrium with the bathing solution, and (iii) the uptake rate is reduced by an additional factor of 0·50 because the bathing solution is unstirred. The over-all correction factor for the CaEGTA concentration due to these factors is (0·65–0·78) (0·57) (0·50) = 0·18–0·22. This agrees as well as can be expected with the experimentally observed value of 0·25.

Relaxation of intact fibres

When the concentration of ionized Ca in the myofilament space, $[Ca]_i$, exceeds the contraction threshold, the myofilaments develop force. At concentrations between the threshold (pCa 6·7) and the plateau (pCa 6·0) of the force–Ca relation, the Ca bound to the myofilaments, $[Ca_{MF}]$, is a linear function of pCa (Weber & Herz, 1963), so that

$$[Ca_{MF}] = K_1 \log_{10} [Ca]_i + K_2, \qquad (4)$$

where K_1 and K_2 are constants. Since the difference in $[Ca_{MF}]$ at pCa 6·0 and at pCa 6·7 is 10^{-4} M (Weber & Herz, 1963; Ebashi & Endo, 1968), the value of K_1 is $10^{-4}/(6{\cdot}7-6{\cdot}0) = 1{\cdot}4 \times 10^{-4}$ M. The value of K_2 depends

on the amount of Ca that binds to the myofilaments at concentrations below the contraction threshold.

The time required for the SR to remove Ca from the myofilaments during the relaxation of intact fibres can be estimated from eqn. (2) and (4). Differentiating eqn. (4), noting that $d[Ca_{MF}]/dt \approx -v$, and making use of eqn. (2) gives

$$\frac{d}{dt}[Ca]_i = \frac{\xi}{K_1 \log_{10} e}[Ca]_i^2. \tag{5}$$

Integrating this equation with the boundary condition that $[Ca]_i = 10^{-6.0}$M at $t = 0$ gives the time (in sec) required to reduce the concentration of ionized Ca from pCa 6·0 to any value of $[Ca]_i$,

$$t = \frac{K_1 \log_{10} e}{\xi}\left(\frac{1}{[Ca]_i} - \frac{1}{10^{-6}}\right). \tag{6}$$

Taking the value of K_1 derived above, and noting that $\xi \geqslant 1{\cdot}4 \times 10^3$ sec^{-1}, eqn. (6) becomes

$$t \leqslant 0{\cdot}04\left(\frac{10^{-6}}{[Ca]_i} - 1\right). \tag{7}$$

In the present solutions, the steady force was half-maximum at pCa 6·3–6·4. According to eqn. (7), this level is reached in less than 40–60 msec. The half-relaxation time of isometrically contracting, tetanically stimulated, fibres under similar conditions (20° C, sarcomere length 2·2μ) is about 40 msec. Therefore the present data indicate that the uptake of Ca by the SR at the low concentrations associated with relaxation is fast enough to account for relaxation. This is an important result because the velocity of Ca uptake by preparations of isolated fragmented SR *in vitro* is too slow to account for the physiological rate of relaxation (Ebashi & Endo, 1968).

If ξ were much greater than the lower limit given by the present experiments, myofilament Ca could be taken up faster than the observed rate of relaxation. Relaxation in this case would reflect another slower process (e.g. dissociation of calcium from the myofilaments, breaking of cross-bridges, etc.).

We have assumed that the force–Ca relation and the value of ξ for intact fibres is close to that of the skinned fibres in the present solutions. This may not be the case, since the Mg^{2+} concentration in intact fibres is apparently much higher than that in the present solutions (Ford & Podolsky, 1972), which would shift the force–Ca relation toward higher Ca ion levels (Ebashi & Endo, 1968) and possibly increase the value of ξ (Ford & Podolsky, 1972). However, the integrated form of eqn. (5) shows that both these effects tend to reduce the time required to lower the concentration of ionized Ca from the plateau to the half-maximum point, so that under physiological conditions the SR would be expected to take up the myofilament Ca faster than under the conditions used here.

End of the delay

The delay generally ended in the more strongly buffered solutions ($[EGTA]_0$ = 0·5 and 2·0 mM) when the Ca taken up by the SR, averaged over the fibre volume, was about 2 mM. Since this is close to the maximum amount of Ca that can be taken up by the SR from the solution, the uptake rate, v, probably slows down at this time and the Ca level within the fibre begins to approach the equilibrium value (eqn. (1)).

The capacity of fibre segments to accumulate 2–3 mM-Ca agrees reasonably well with the value (> 1 mM) found by adding droplets containing a $CaCl_2$ solution to skinned fibres in oil (Podolsky, 1968), which indicates that the bathing solutions used in the present study are not deficient in components essential for Ca accumulation. The capacity found here is also of the same order as that reported for isolated SR fragments (Weber, Herz & Reiss, 1966).

The total capacity of the SR for Ca is somewhat greater than that measured by ^{45}Ca uptake since at the start of the experiment the SR contained 0·6 mM (Winegrad, 1968) to 0·8 mM (Curtis, 1970) unlabelled Ca. This endogenous Ca probably remained in the SR during equilibration with the 0·1 mM-EGTA solution (see Fig. 2) and also in the buffered Ca solutions.

If filling of the SR were the only process that terminated the delay phase, force would develop monotonically. However, the patterns of force development seen in Fig. 5*c*, *d*, and *e* indicate that the end of the delay is often associated with release of Ca from the SR. This suggests that the contraction threshold is close to the Ca concentration required to trigger the release of accumulated Ca from the SR (Ford & Podolsky, 1970). Since the release process is intrinsically regenerative, it has the potentiality of propagating through the fibre volume. The intervention of this process, which is described in detail in the following paper, probably explains the variations seen in the duration of the delay phase for different preparations in solutions having the same composition (Table 1).

If the capacity of the SR to take up Ca were unusually low in one region of the fibre (due, perhaps, to local damage during dissection) the calcium concentration would approach the equilibrium value, and possibly trigger release of accumulated Ca, sooner in that region than in the rest of the fibre. If, in addition, propagation of the release process occurred, the delay phase would end before the capacity of the entire SR for calcium had been reached. Propagation is more likely when the concentration of EGTA in the myofilament space is relatively low, which might explain why the delays in solutions at pCa 6·2 were shorter than expected on the basis of SR filling rate when $[EGTA]_0$ = 0·1 mM, but not when $[EGTA]_0$ = 0·5 and 2·0 mM.

A force pattern consisting of repetitive spikes can also be explained if force development is associated with calcium release from the SR, since

the release process would be expected to create additional capacity in the SR for Ca accumulation and, therefore, another delay phase. Repetitive spikes were observed very infrequently, however, probably because the SR was able to reaccumulate quickly the Ca that had been released. Such rapid reaccumulation would take place if the concentration of the released Ca exceeded that of the EGTA, since this excess Ca would be free and available for uptake.

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