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CALCIUM CONTENT AND EXCHANGE IN FROG SKELETAL MUSCLE

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SUMMARY

1. Calcium content and exchange in frog ELD IV muscle were examined employing the efflux technique.

2. Muscle calcium was found to exchange with four time constants, 21·5 sec, 2·7, 32 and 1244 min.

3. All calcium was found to be exchangeable with more than half the total amount residing in an extracellular compartment.

4. Results obtained from ELD IV muscles and single fibres were identical.

5. Muscle calcium content was found to remain constant up to 20 hr *in vitro*.

6. Extra exchange of calcium occurs upon contraction. This extra exchange appears to occur in the most slowly exchanging component.

7. The data are discussed in relation to morphological and autoradiographic findings and a model of calcium exchange in skeletal muscle is presented.

INTRODUCTION

Subcellular compartmentalization of calcium is the key to the control of contraction and relaxation in skeletal muscle (Weber & Murray, 1973). There has been substantial progress in understanding of calcium binding by the protein components of the contractile system (Fuchs & Briggs, 1968) and by isolated fragments of the muscle membrane system (Martonosi, 1971). Synthesis of results from these isolated systems into understanding of the performance of intact muscle cells requires a quantitative picture of calcium dynamics in functioning cells. Recent autoradiographic studies (Winegrad, 1968, 1970) have pointed to the need to extend the classical studies of calcium exchange in skeletal muscle.

Gilbert & Fenn (1957) analysed $^{45}Ca^{2+}$ influx into frog skeletal muscle and developed a model in which Ca^{2+} was distributed among a variety of

extracellular and surface pools, one intracellular pool, and an inexchangeable pool. Shanes & Bianchi (1959*a*, *b*) reported studies of $^{45}Ca^{2+}$ efflux in a series of papers, two of which are cited. These workers found Ca^{2+} in frog muscle to be located in two pools: one surface pool exchanging with a time constant of 10 min and an intracellular pool with a much longer time constant variously reported from 200 (Bianchi & Bolton, 1974) to 500 min (Shanes & Bianchi, 1959*a*). Curtis (1970) studying $^{45}Ca^{2+}$ efflux from single fibres found three components of wash-out. In the present work we have sought to minimize problems of experimental design through the following methods: (1) the efflux method was used; (2) the muscles were loaded with $^{45}Ca^{2+}$ for long periods; (3) the first wash-out samples were collected earlier in time than in previous studies; (4) the last wash-out samples were collected later in time than in previous studies; (5) objective non-linear least squares procedures were used for parameter estimation after initial estimates were obtained by graphical analysis.

By employing this methodology in our work we have concluded that there are four components of $^{45}Ca^{2+}$ wash-out in skeletal muscle. In addition, we report that all muscle calcium is exchangeable on a scale of hours. These findings are discussed in terms of a model of calcium exchange in skeletal muscle. Portions of this work have been reported previously (Picken, Kirby & Lindley, 1974).

METHODS

Frogs, *Rana pipiens*, were obtained from Mogul-Ed (OshKosh, Wisc., U.S.A.) and were maintained in the laboratory at room temperature. They were fed twice weekly on mealworms at which time the water in the tank was changed.

Ringer for these experiments consisted of (mM): 117 NaCl, 2·5 KCl, and 2·0 $CaCl_2$. The solutions were buffered to pH 7·0 with 2·5 mM-NaH_2PO_4. Curare, 10^{-5} g/ml. was added to all solutions. The solutions in which the muscles were equilibrated with $^{45}Ca^{2+}$ were of the same composition with the exception of the presence of $^{45}Ca^{2+}$ (ICN Pharmaceuticals, Inc.).

The efflux chamber used in these experiments was identical to those of Curtis (1970) and Moore (1969). This type of chamber is based on a principle originally introduced by Hodgkin & Keynes (1955), the advantage being that the effluent is collected only from the centre of the tissue with any solution passing by the ends being discarded. Our chamber contained platinum stimulating electrodes for electrical stimulation of the muscles.

A typical experiment was performed in the following manner. The frog was decapitated, the lower legs skinned and the extensor digitorum longus, ELD IV muscle ('toe muscle') dissected in frog Ringer. The muscle was carefully cleaned in Ringer under 30× magnification until no visible connective tissue remained. Muscles containing damaged fibres were rejected. The muscle was attached to a manipulator and stretched to a length approximating 1·1 times that in the body. The muscle was then transferred into the influx chamber which was adjacent to the efflux chamber, and submerged in approximately 1 ml. frog Ringer containing $^{45}Ca^{2+}$. At

the end of the equilibration period, from 10 min to 17 hr, the muscle was moved to the efflux chamber. For influx periods lasting more than 1 hr, the influx chamber was covered with Parafilm and the solution in the chamber was equilibrated with 100 % oxygen.

Upon transfer to the efflux chamber and following careful positioning of the muscle, the flow of Ringer containing no $^{45}Ca^{2+}$ was started and samples were collected. Positioning and flow adjustment normally required 2–3 min so that in most experiments the first sample collected was in the third min after the wash-out had begun. Approximately 1 ml./min passed through the central effluent duct. One min samples of the effluent were collected each min for the first 10 min, then one sample/min every 3 min for the next 9 min, every 5 min for the next 20 min, every 10 min for the next 1 hr, then one sample/min every 20–30 min for the rest of the experiment. Wash-out time lasted from 30–600 min. Wash-outs were conducted at room temperature (22° C). These experiments were performed at various times during the year. Since calcium content is known to vary seasonally (Bianchi, 1968), we have noted in the text where appropriate when the experiments were done.

To record the first 3 min of wash-out the 'dipping technique' was employed, and other toe muscles, equilibrated with $^{45}Ca^{2+}$ for 3 hr, were used for this study. The tendinous ends were trimmed and the muscles were attached to monofilament plastic suture or fine platinum wire and dipped into a series of beakers. Visibly damaged muscles were discarded. Separate wash-outs were done for the plastic or wire holders and it was found that 95 % of the $^{45}Ca^{2+}$ adhering to these structures was removed within the first 10 sec, this amount being less than 5 % of the total isotope present during an actual experiment. The muscles were gently blotted before they were transferred to the first beaker to remove any droplets that might have adhered to the muscle surface; each experiment was carried out for 10 min.

At the conclusion of a wash-out experiment the muscle was removed from the efflux chamber, weighed on a Cahn electrobalance and then dissolved in 1 ml. either concentrated nitric acid or Soluene (Packard). After 12 hr an aliquot of this solution was withdrawn, suspended in scintillation cocktail, and counted to determine $^{45}Ca^{2+}$ left in the muscle at the end of the experiment. In the usual experiment the effluent was collected in a scintillation vial (Packard) to which was added 7·5 ml. following mixture: 1 part Triton-X 100 (Research Products International) to 2 parts toluene (RPI) by volume and 8 g 2·70 pre-blend PPO (RPI)/l. All samples were counted on a Packard Tri-Carb Scintillation counter for 10 min each at least three times. Counting efficiency varied less than 5 % regardless of the amount of $^{45}Ca^{2+}$ in any given sample.

Total chemical calcium determined by atomic absorption spectrophotometry (*AAS*) was obtained from similar ELD IV muscles. Due to the small size of the ELD IV muscle (1–3 mg), seven to ten muscles were grouped following dissection, cleaning, blotting, and weighing. The procedure was similar to that employed by Borys & Karler (1971). The muscles were placed in glass crucibles and ashed at 600° C for 4 hr in a muffle furnace. The ash was dissolved in 0·5 ml. HCl and then diluted with lanthanum and strontium to final concentrations in the sample of 0·20 mM-HCl, 25 mM-La^{3+}, and 100 μM-Sr^{2+}. The samples were analysed for us by Dr Charles MacFarland on an Instrumentation Laboratory Model 153 atomic absorption spectrophotometer.

Treatment of the data

From the efflux results, we constructed desaturation curves by numerical integration of the efflux curve according to the trapezoidal rule. The value of the desaturation curve is twofold: the 'noise' is smoothed by the integration, and

the components are weighted according to pool size. In efflux plots a further weighting according to rate constant for exchange complicates the resolution of slow components.

The desaturation curves were further analysed in that estimates of time constants and pool sizes were made from the plot. These values were then refined by computer non-linear least squares regression fit (Newton–Raphson method). The pool sizes were corrected for lack of specific activity equilibrium. In order to compute fluxes in p-mole/cm^2 per second, average muscle surface membrane area was calculated using 34 μm as an average fibre diameter (Mr Robert Anderson furnished us with the muscle sections prepared according to the Karnovsky (1965) method), fibre density = 1·06, and 15% extracellular space by volume.

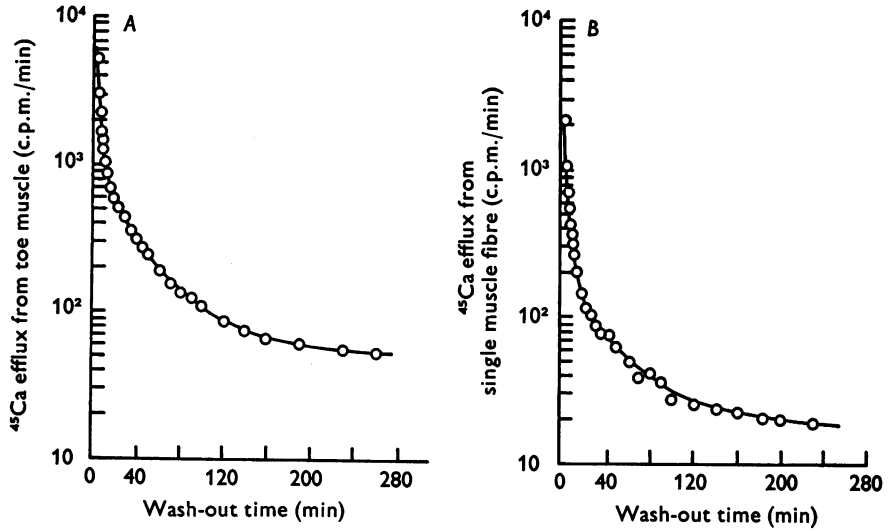

Fig. 1. $^{45}Ca^{2+}$ efflux from frog skeletal muscle (c.p.m./min per milligram). *A*, whole ELD IV muscle; *B*, single fibre from semitendinosus muscle. Points are experimentally obtained, the lines are calculated as the sum of three exponentials. See text for complete equations and discussion.

RESULTS

Time constants and pool sizes in frog ELD

Fig. 1*A* is a plot of the $^{45}Ca^{2+}$ efflux as a function of time. The curved line through the points was calculated from the equation:

$$\text{c.p.m./min} = 10^4\,(3{\cdot}0e^{-t/2{\cdot}2} + 0{\cdot}08e^{-t/35} + 0{\cdot}0062e^{-t/1149}).$$

A second independent method can be used for obtaining this final time constant. If the final point on the efflux plot (c.p.m./min per milligram or dA/dt) is divided into the final counting rate of the muscle (c.p.m./mg = A), the time constant can be obtained ($T = A/(dA/dt)$) or 1100 min. This second method of obtaining the time constant of the slowest exchanging component is especially important because it indicates that all of the remaining $^{45}Ca^{2+}$ in the muscle at the end of the experiment can be accounted for by this final exponential component.

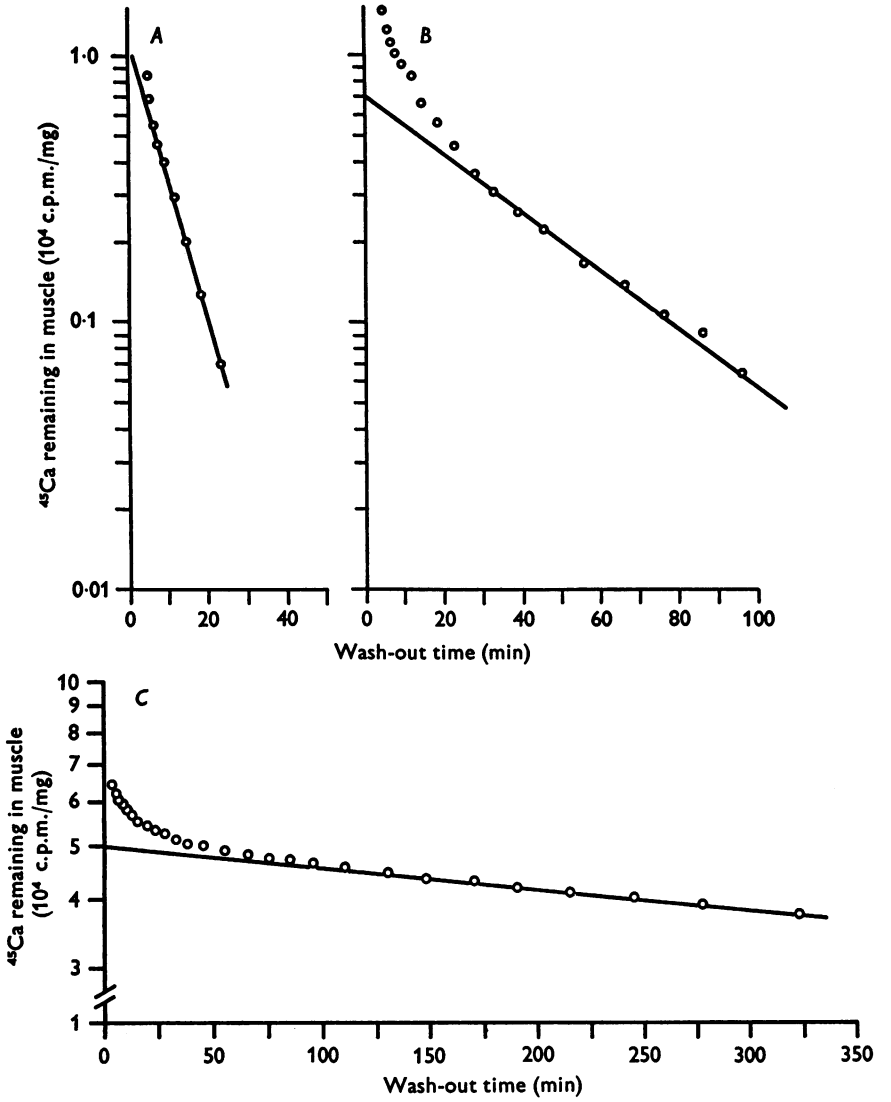

Fig. 2. Desaturation plot of $^{45}Ca^{2+}$ content of ELD IV muscle. *C*, total $^{45}Ca^{2+}$/mg tissue, time constant of 1152 min. *B*, $^{45}Ca^{2+}$/mg tissue after subtraction of the most slowly exchanging component from the original plot, time constant of 40 min. *A*, $^{45}Ca^{2+}$/mg tissue after subtraction of the intermediate component, time constant of 5·5 min. Points are experimentally obtained, the lines are calculated using objective non-linear least squares procedures. See text for detailed discussion of data treatment.

The $^{45}Ca^{2+}$ wash-out from another toe muscle is represented in the desaturation plot in Fig. 2*C*. If the points before the first 120 min are neglected, the remaining points can be fitted to a straight line, in this case, with a time constant of 1152 min. The amount of $^{45}Ca^{2+}$ in this component can be obtained by extrapolating back to zero time. Because the muscle was loaded with $^{45}Ca^{2+}$ for a shorter period (14 hr) than the final time constant of calcium exchange (over 19 hr) a correction factor was used to obtain the $^{45}Ca^{+}$ content at equilibrium. The equation used as $A = A_m/(1 - e^{-m/t})$ where A is equilibrium pool size, A_m is pool size at loading duration = m and T is the time constant. (The amount of calcium in this component was obtained by dividing the $^{45}Ca^{2+}$ content under steady-state conditions by the calcium specific activity of the load solution. The pool size for this example was 0·77 m-mole Ca^{2+}/kg wet muscle tissue, assuming independent wash-out of each component.)

By 'peeling off', that is by subtracting the slowest exchanging component from the original desaturation plot, the earlier components of the calcium wash-out can be obtained. Fig. 2*B* shows the results of this manipulation. It is clear that if the points before the first 10 min are neglected, the remaining points can be fitted with a straight line. This intermediate component exchanged with a time constant of 40 min and contained 0·06 m-mole Ca^{2+}/kg tissue in this example. Specific activity equilibrium had been reached in this faster exchanging component so a correction factor was not used. By subtracting once more, the fastest exchanging component can be obtained and is represented in Fig. 2*A*. Here this component exchanged with a time constant of 5·5 min and contained 0·27 m-mole Ca^{2+}/kg tissue.

TABLE 1. Calcium exchange in intact toe muscle after first 3 min of wash-out

	Time constant, min	Pool size, m-mole/kg	Flux, p-mole/cm² sec
Component 1	2·7 ± 0·02	0·20 ± 0·02	1·27
Component 2	32 ± 4·00	0·09 ± 0·01	0·048
Component 3	1244 ± 85·00	0·54 ± 0·06	0·0076
		Total = 0·83	

Means ± S.E. of mean for nine experiments.

Means and S.E. mean of nine such experiments are tabulated in Table 1. The steady-state calcium fluxes from each component are also shown. The total calcium in these three components is 0·83 m-mole-Ca^{2+}/kg tissue. These experiments were performed using muscles from summer frogs, June until September.

In summary, the calcium exchange process in whole toe muscles after

the first 3 min of the wash-out can be described by the sum of three exponentials, with time constants varying from several min to over 1000, as stated by the following equation:

$$\text{calcium content (m-mole/kg)} = 0{\cdot}2e^{-t/2{\cdot}7} + 0{\cdot}09e^{-t/32} + 0{\cdot}54e^{-t/1244}.$$

Single muscle fibre wash-outs

In order to ascertain that toe muscle calcium wash-outs were similar to individual cell wash-outs, $^{45}Ca^{2+}$ efflux experiments were done using single muscle fibres. The possibility existed that the three components so far described actually originated from different rates of calcium exit from fibres of different diameters (range 18–55 μm).

Single muscle fibres were obtained from the semitendinosus muscle and the procedure for these experiments was identical to that employed for the muscle efflux studies after the first 3 min of wash-out. Fig. 1*B* illustrates a typical efflux curve from a single fibre; as in Fig. 1*A* the curved line through the points was calculated from an equation with time constants in this particular fibre of 3·6, 36 and 1008 min for fast, intermediate, and slow components respectively. For purposes of comparison, flux from the most slowly exchanging component was 0·005 pm/cm^2 per second in single fibres, 0·0076 pm/cm^2 per second in whole muscle. Six separate single fibre wash-outs gave similar results. Therefore, we felt justified in using toe muscles for most of the experiments instead of single fibres because of the greater ease of dissection and handling.

Muscle calcium content as a function of time after dissection

Experiments to determine muscle calcium content as a function of time spent *in vitro* consisted of the following procedure: immediately after a dissection, one toe muscle of a pair was labelled for 3 hr in $^{45}Ca^{2+}$ containing Ringer, followed immediately by a wash-out study. The second muscle of this pair was left 20 hr in Ringer and the following morning equilibrated for 3 hr in $^{45}Ca^{2+}$-containing Ringer, following which a wash-out experiment was performed. The only difference was a 5% increase in the estimated Ca^{2+} content of the slowest exchanging component of the muscle soaked for 20 hr. The time constants of the components are the same. These results indicate that the calcium content of toe muscle is not significantly altered up to 20 hr *in vitro* if handled and treated as described in the Methods.

Short load times were examined in a second group of experiments. Three different muscles were equilibrated in $^{45}Ca^{2+}$-containing Ringer, for 15, 90 and 215 min respectively. After the labelling period, a complete $^{45}Ca^{2+}$ wash-out experiment was carried out for each muscle. The $^{45}Ca^{2+}$

desaturation plots are shown in Fig. 3. The time constants and the component sizes of the most slowly exchanging component (corrected to specific activity equilibrium) were 1188 min, 1·54 m-mole Ca^{2+}/kg; 1123 min, 1·74 m-mole Ca^{2+}/kg; 1296 min and 1·5 m-mole Ca^{2+}/kg for the 15, 90, and 215 min influx times respectively. The close agreement in time constants and corrected component sizes verifies the previous results that the parameters described do not vary as a function of time the muscles spend *in vitro*. These experiments were carried out in early winter (December) and the increased calcium content of these muscles over summer muscles is reflected in the slowest exchanging component size above, an amount of calcium approximately triple that found in the summer.

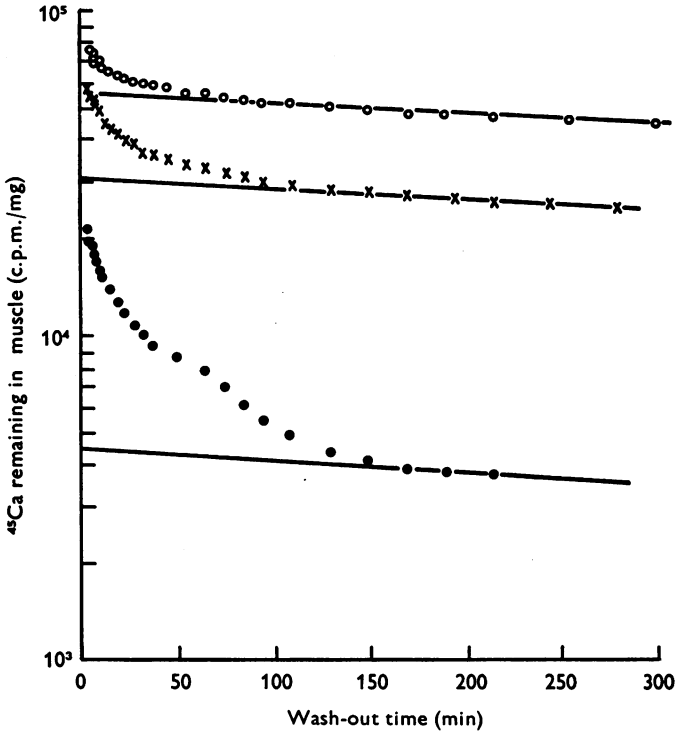

Fig. 3. Effect of load time on calcium wash-out. The three $^{45}Ca^{2+}$ desaturation curves represent the $^{45}Ca^{2+}$ wash-out from three different toe muscles, soaked in $^{45}Ca^{2+}$ containing Ringer for 15 (●), 90 (×), and 215 min (○) respectively. The time constant of exchange for the slowest component and the calcium content corrected for specific activity equilibrium are given in detail in the text. See text for complete discussion.

Extracellular space exchange

These experiments were carried out by means of the 'dipping' method and by atomic absorption spectrophotometry.

A large amount of calcium was found to exchange in less than 1 min, Fig. 4. Analysis of the graph indicates that two-thirds of a pool containing

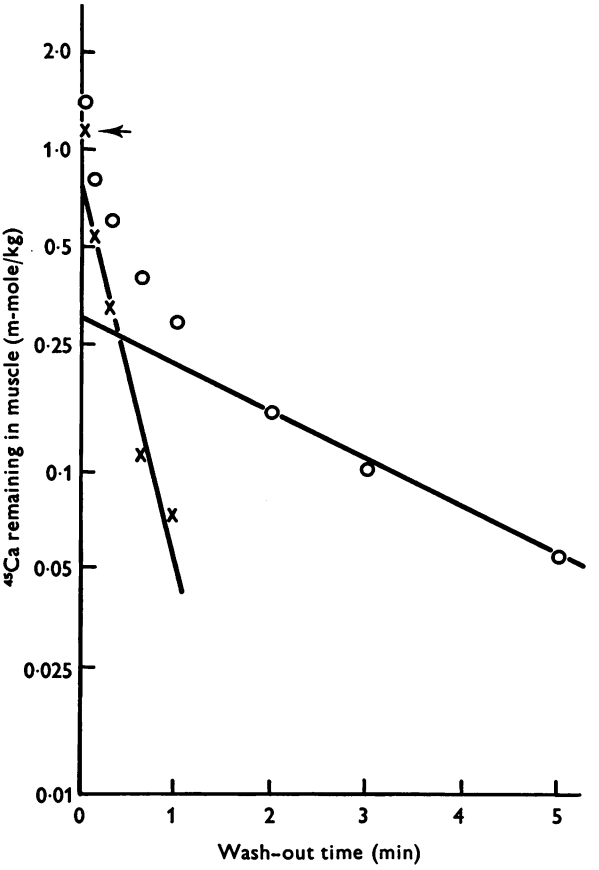

Fig. 4. The open circles represent the exchange of calcium in m-mole/kg from an intact toe muscle (mean values for five different muscles) during the first 10 min of a wash-out. The extrapolated line connecting the points after the first 2 min represents calcium exchanging with the fastest intracellular component. By the method of peeling off the faster component in time (×) is thought to represent calcium exchange with the extracellular space. The extrapolated line is the slowest exponential component of a series for the extracellular space wash-out and is described by a time constant 21·5 sec and a calcium content of 0·80 m-mole Ca^{2+}/kg. The difference between the total amount of calcium in the extracellular space, 1·13 m-mole Ca^{2+}/kg (×←) and the slowest extracellular space component, is that calcium exchanging faster in the series of additional exponentials.

1·13 m-mole Ca^{2+}/kg exchanged with a final time constant of 21·5 sec. These data are consistent with diffusion from a cylinder radius 150 μm of a substance with a diffusion coefficient 2×10^{-6} cm^2/sec (Milligan, 1965; see Jacobs, 1967).

A second method of analysing the extracellular calcium content in normal muscles employed the use of atomic absorption spectrophotometry. Ten muscle pairs were used: one member from each pair was analysed for total calcium after transfer from ordinary Ringer and blotting. The remaining members of each pair were analysed after a 3 min soak in calcium-free Mg^{2+}-containing Ringer. The total calcium content was 2·35 m-mole/kg for controls and 1·18 m-mole Ca^{2+}/kg for muscles soaked for 3 min in calcium-free Ringer; the amount lost in 3 min was 1·17 m-mole Ca^{2+}/kg. Although the fastest exchanging intracellular component should have lost 0·14 m-mole/kg in a 3 min wash, leaving 1·03 min Ca^{2+}/kg in the extracellular space, this value is very close to that obtained by the isotopic wash-out method (1·13 m-mole Ca^{2+}/kg), and the two approaches together strongly validate one another.

Given a calcium concentration of 2 mM in Ringer, approximately 0·34 m-mole Ca^{2+}/kg muscle tissue should be present in the extracellular fluid. This is considerably less than the amount of calcium observed, so the difference must be bound to extracellular proteins, i.e. collagen, muscle surface membrane and walls of the transverse tubular system.

TABLE 2. Calcium content of frog toe muscle

Location	m-mole/kg
Extracellular	1·13
Intracellular	0·83
Total exchangeable	1·96
Total (*AAS*)	2·00
Difference	−0·04

Total muscle calcium

The exchangeable calcium determined by $^{45}Ca^{2+}$ efflux studies can now be divided into two major divisions, extracellular and intracellular: the total exchangeable calcium in normal muscle of summer frogs is 1·96 m-mole Ca^{2+}/kg tissue.

Total calcium concentration was independently determined by atomic absorption spectrophotometry. Because of the small size of the muscles, each determination required the use of ten toe muscles. The results are summarized in Table 2. Total calcium of summer frogs by this method was 2·00 m-mole Ca^{2+}/kg tissue. Total exchangeable calcium and total calcium determined by spectrophotometry were almost the same. These

results indicate that all of the toe muscle calcium can be accounted for in the exchangeable fractions.

Increased calcium efflux associated with muscle activity

During these experiments, the muscles were stimulated at one, two and five pulses/sec for 1 min and the entire stimulated efflux was collected; the results are summarized in Table 3. It is important to note that the muscles were contracting isometrically so that no gross muscular movement occurred. It is apparent from these data that the calcium efflux increased approximately nine, thirteen and twenty-three-times over the resting calcium efflux values at simulation frequencies 1, 2 and 5 c/s respectively. Because the efflux per twitch decreased at higher frequencies of stimulation, the amount of calcium exchanging appeared to be a 'saturable' phenomenon. It was also noted that at much greater stimulation frequencies (25/sec) or during 100 mM-K^+ contractures, the amount of calcium efflux was approximately the same as at 5/sec, indicating the 5/sec values to be close to a maximum.

TABLE 3. Calcium efflux associated with muscle activity

Stimulation rate	Total efflux p-mole/cm² per second	Ratio to basal value	p-mole/cm² per twitch increase over rest efflux
rest	0·0076	1·0	—
1/sec	0·066	8·7	0·059
2/sec	0·096	12·6	0·047
5/sec	0·175	23·0	0·035

The absolute magnitude of the stimulated efflux decreased in parallel fashion with the loss of isotope from the slowest exchanging component (Fig. 5). If the stimulated efflux points are connected with a straight line, the time course of the drop-off in activity was the same as that estimated for the loss of activity from the slowest exchanging component. This parallel decrease in activity would be expected if all the label remaining in the muscle was that calcium exchanging with the slow component.

A kinetic analysis of the onset and relaxation of the stimulated flux was also done approximately 300 min after the wash-out had started. Five samples per minute were collected during a 1 min stimulation period (2 pulses/sec) and for 1 min thereafter, followed by 1 min collections for an additional 3 min. The results of one such experiment are shown in Fig. 6. Additional activity appeared almost immediately after stimulation began, the calcium efflux rising to a peak value approximately fifteen times the efflux observed at rest. When muscle activity ceased, calcium efflux relaxed to basal values with two time constants, 24 sec and 2·1 min.

This strongly suggests that during muscle activity, the faster exchanging intracellular component is partially relabelled, its subsequent wash-out being observed by a time constant appropriate for this component. The time constant of the first part of the relaxation phase is characteristic of extracellular space wash-out. The time course of appearance of this activity emerging at the muscle surface was calculated (Carslaw & Jaeger, 1959) assuming that the efflux of activity into the extracellular space rose

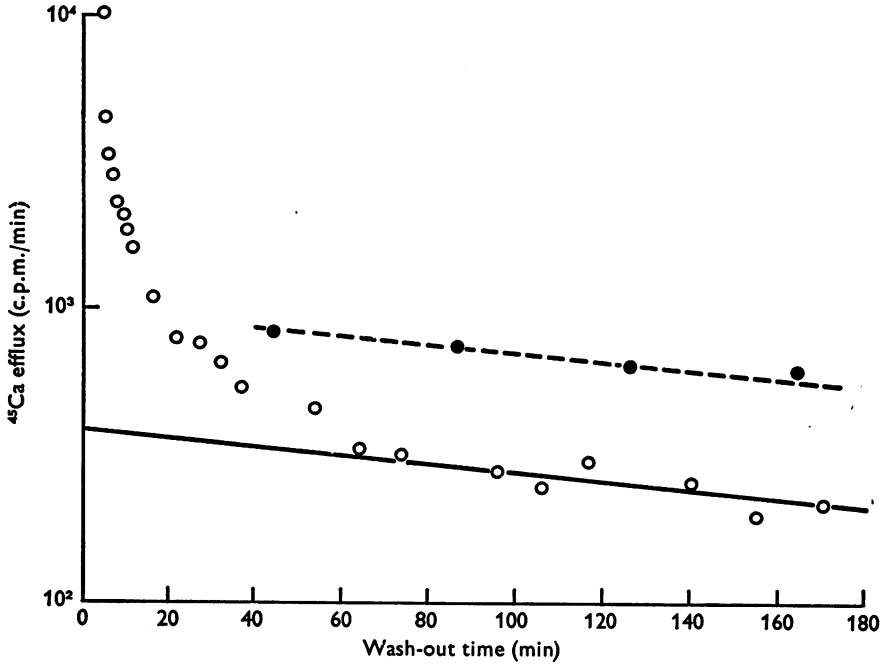

Fig. 5. $^{45}Ca^{2+}$ efflux upon stimulation. The elevated points (●) are the increased calcium efflux for a 1 min interval with a muscle stimulation frequency of 1 Hz. (○) represent unstimulated efflux.

immediately to a maximum value (fifteen times resting efflux in this case) upon muscle stimulation and was maintained at this level throughout the stimulation period. The dashed line in Fig. 6 represents the predicted values for the emergence of additional label. It is a fair fit to the observed values, confirming that the additional calcium associated with muscle activity was delivered immediately to the extracellular space upon stimulation and subsequently appeared in the bathing media with time constants characteristic of the extracellular compartment.

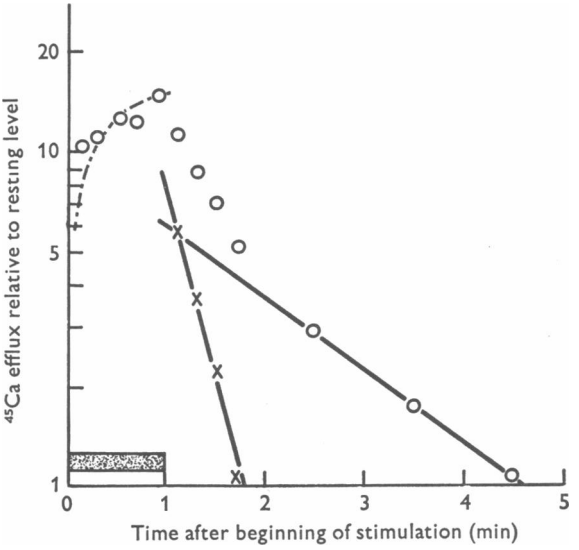

Fig. 6. Onset and relaxation of the increased calcium efflux associated with muscle activity as a function of time. The data are expressed as the number of times the stimulated efflux increased over the rest values. The relaxation phase declined with two time constants, 24 sec and 2·1 min, respectively. The dashed line in the rising phase of the plot represents the predicted values for the appearance of additional activity at the surface of the muscle, based on the assumption that the increased efflux of activity into the muscle interstitium immediately rose to maximal values (fifteenfold) at the onset of muscle activity and appeared with time constants of the extracellular space wash-out. The dark bar represents the time during which stimulation occurred.

DISCUSSION

We have presented data which indicate that frog skeletal muscle calcium is localized in four pools, one extracellular and three intracellular. That the ELD IV muscle is an adequate substitute for single fibres is clear in that ELD IV wash-out is not different from $^{45}Ca^{2+}$ wash-out from semitendinosus single fibres. The importance of the slowest exchanging component, which has received little attention in previous studies, became clear only after long loading periods, up to 17 hr. Our data indicate that the long loading periods *in vitro*, under our conditions, have no significant effect either upon the time constants of exchange or on the total calcium content. Therefore, we feel justified in our selection of the ELD IV as an adequate experimental preparation and we feel that the use of long $^{45}Ca^{2+}$ loading periods has no deleterious effect on the muscles.

With our approach there is a great consistency of wash-out time constants from frog to frog and under varying conditions. We attribute the

lesser consistency in much published work to errors in parameter estimation resulting from improper sample spacing and experiment duration. Under such conditions, the pool sizes will interact with the apparent time constants, e.g. an increased slow pool will cause a shift in an erroneously fast estimate of the time constant towards the true value.

The coefficients and rate constant of the exponential terms can be assigned in a direct correspondence to compartments assumed to be arranged in parallel. However, it seems necessary *a priori* to assume that the extracellular space is in series with the tissue compartment. We examined the alternatives of: (*a*) pairwise correction by the Huxley (1960) formula and (*b*) correction of the 2–3 min component for diffusion in the extracellular space by the approach of MacDonald *et al.* (1974). Both approaches yield substantial corrections to flux parameters, but of opposite signs; pool size corrections also differ significantly. Alternative (*a*) yields only small changes in Pools 1 and 4, a 20% increase in Pool 3, and a 25% decrease in Pool 2.

The Huxley approach, however, is unrealistic for the cylindrical toe muscle, since the superficial muscle fibres exchange through only a short extent of unmixed extracellular space and the internal muscle fibres through a greater extent. In fact, correction of Compartment 2 according to the expressions of MacDonald, Mann & Sperelakis (1974) suggests that the 'true' intracellular pool will be negligibly different from the sum of the zero time intercepts of the three slowest components of the desaturation curve (assuming 150 μm radius of the muscle bundle and a Ca^{2+} diffusion coefficient of 2×10^{-6} cm^2/sec).

We thus conclude that, if the fastest component of Ca^{2+} exchange (1·13 m-mole/kg) is evenly distributed outside the cells, the empirical intercepts of the three slow components are close to the values of the physical pool sizes in the muscle.

The assignment of morphological entities to the components of the $^{45}Ca^{2+}$ wash-out curve is an essential part in understanding calcium compartmentation and metabolism of muscle. Winegrad (1968, 1970) has presented evidence that $^{45}Ca^{2+}$ appears largely in the sarcoplasmic reticulum of muscle and that the reticulum exchanges with two time constants. His data indicate that 0·08 m-mole Ca^{2+}/kg tissue exchange with the terminal cisternae in 5 min with no labelling of the longitudinal reticulum. Our data would suggest that 0·15 m-mole Ca^{2+}/kg would exchange but considering the resolution of the grain counting method and variability between batches of frogs, the agreement is good. In 5 hr Winegrad's data indicate 0·1 m-mole Ca^{2+}/kg exchange with the longitudinal reticulum, a value not different from the 0·096 m-mole Ca^{2+}/kg predicted from our data. Our data showing increased efflux upon stimula-

tion late in the wash-out also suggest that the slowest exchanging component is part of the sarcoplasmic reticulum. Therefore, we feel that the two components of wash-out with time constants 2·7 and 1244 min represent sarcoplasmic reticulum and specifically that the faster component is the terminal cisternae and the slower, the longitudinal and fenestrated collar region. The intracellular locus of the intermediate compartment (with time constant of 32 min) is still unclear; a tentative allocation is the mitochondria. Fig. 7 shows schematically the relative size of the components and the suggested morphological correlation.

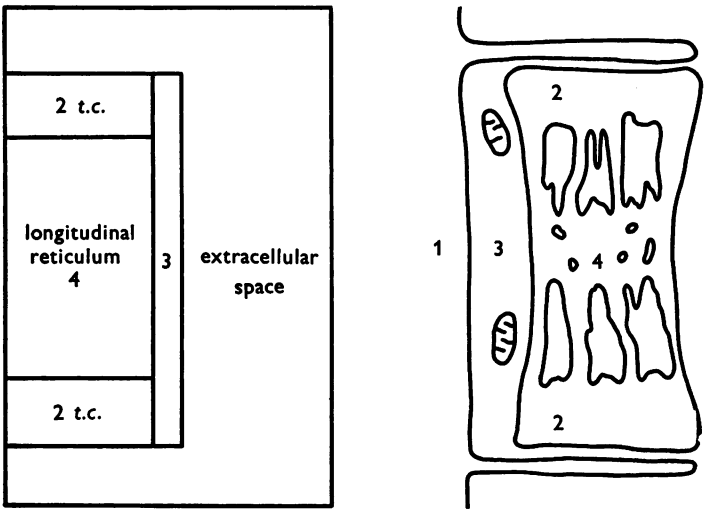

Fig. 7. Diagrammatic representation of the components of calcium wash-out. 1 = extracellular space; 2 = terminal cisternae, *t.c.*; 3 = myoplasm and mitochondria and 4 = longitudinal reticulum. See text for complete discussion.

In conclusion, we have shown that the calcium in frog skeletal muscle is entirely exchangeable on the time scale of our experiments and that more than half of this calcium resides in a pool of 'extracellular' calcium. We have also pointed out the existence of a pool of calcium believed to be cisternal calcium which turns over with an average time constant of 2·7 min. This rapid turnover of a Ca^{2+} pool closely linked to contraction suggests that interpretation of much previous data requires modification.

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