BRIEF COMMUNICATIONS

^{45}Ca AND [^{14}C]EDTA EFFLUX FROM DIALYZED BARNACLE MUSCLE FIBERS

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ABSTRACT ^{45}Ca and ^{14}C-labeled ethylenediamine-*N*, *N'*-tetraacetic acid (EDTA) effluxes were measured in internally dialyzed barnacle muscle fibers. In ^{45}Ca experiments the internal ionized ^{45}Ca was fixed at 0.2 μM with ethyleneglycol-bis-(beta-aminoethylether)-*N*, *N'*-tetraacetic acid (EGTA). The ^{45}Ca efflux was found to increase with internal CaEGTA from 0.05 pmol/cm^2 · s (CaEGTA = 0.02 mM) to 5.0 pmol/cm^2 · s (CaEGTA = 9.6 mM). To determine whether or not most of this increase in efflux was due to the exit of undissociated CaEGTA, comparable experiments were performed with Ca-[^{14}C]EDTA. Over the same range of internal calcium as studied in the ^{45}Ca experiments, the Ca-[^{14}C]EDTA efflux was no more than 12% of the ^{45}Ca efflux. We conclude that the exit of undissociated ^{45}Ca cannot account for most of the ^{45}Ca efflux nor can it account for the dependence of ^{45}Ca efflux on internal CaEGTA. The experiments also demonstrated the existence of an endogenous pool of calcium, of 0.43 mmol/kg (about half the total calcium), which remained unexchanged during dialysis.

INTRODUCTION

Previous studies of calcium efflux from barnacle muscle fibers have utilized a microinjection technique (Ashley et al., 1972; Russell and Blaustein, 1974). These results have generally supported the hypothesis, developed from earlier work on squid axons (Baker et al., 1967; Blaustein and Hodgkin, 1969) that a significant fraction of calcium efflux is coupled to sodium influx. However the ionized calcium concentration in sarcoplasm is not accurately known. In view of the recent evidence in squid axons that both the magnitude of calcium efflux and the extent of calcium-calcium and calcium-sodium exchange depend upon the level of ionized calcium in the axoplasm (Blaustein and Hodgkin, 1969; Mullins and Brinley, 1974, 1975) definitive control of internal calcium would seem to be important in experiments on muscle fibers.

Such control is feasible with the use of the internal dialysis technique and organic calcium chelators which can serve as buffers of the ionized calcium concentration.

In the experiments reported here, the calcium efflux was measured with the use of radioactive calcium in conjunction with either EGTA (ethyleneglycol-bis-(beta-amino-ethylether)-*N*, *N'*-tetraacetic acid) or EDTA (ethylenediamine-*N*, *N'*-tetraacetic acid). Since the intracellular organelles in muscle fibers contain a relatively large amount of calcium, which can exchange with the radioactive calcium in the dialysis medium during dialysis, the time required to reach isotopic equilibrium should be longer with lower concentrations of chelated calcium in the dialysis medium. This expected result was fully confirmed in the experiments to be reported. In addition the plateau or steady-state level of calcium efflux was found to increase with increasing total buffer concentration. This result was somewhat unexpected, since in squid axons, the efflux of calcium appears to depend mainly upon the internal ionized calcium concentration and not upon the concentration of chelated calcium. The simplest explanation of the present results obtained in barnacle muscle fibers, a nonspecific leak of chelated calcium, was rendered highly unlikely by the results of parallel experiments in which the leak of calcium in the buffered form was measured with ^{14}C-labeled chelator. The efflux of buffered calcium was less than 12% of the total calcium efflux.

During these investigations, additional data were obtained which permitted estimation of the amount of endogenous fiber calcium which did not equilibrate isotopically with the tagged calcium in the dialysate.

METHODS

Apparatus

The procedures and equipment used in the internal dialysis technique for single muscle fibers have been described previously (Brinley and Mullins, 1967; DiPolo, 1972). The depressor scutum lateralis or, more frequently, the depressor scutum rostralis was isolated from the barnacle *Balanus aquila* (Pilsbry).

A significant improvement in the technique was the use of dialysis capillaries made of porous cellulose rather than porous glass. The properties of this material have been described elsewhere (Brinley and Mullins, 1974). The outside diameters of the capillaries were usually 140 or 196 μm. The perfusion rate of the dialysate was about 1.1 μl/min. The flow rate of the external bathing solution was 1–1.2 ml/min. Samples were collected at 2- or 4-min intervals with an automatic fraction collector and counted in either a low background Geiger counter or a beta scintillation counter. Efflux of ^{45}Ca or [^{14}C]EDTA across the sarcolemma was calculated from the activity of the tracer appearing in the external bath and a knowledge of the internal specific activity. In making these calculations it was assumed that the fibers were cylindrical; the surface area of the membranes bounding the clefts was not taken into account.

The composition of the external bathing solution was (millimolar): K, 10; Na, 460, Mg, 32; Ca, 0; Cl, 534; EDTA, 0.1; TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid), 5; pH 7.6. The basic internal solution was (millimolar): K, 93; Na, 15, Cl, 29; aspartate, 79; glucose, 233; glycine, 233; KBES, 19; HBES, 29 (BES, *N*, *N'*-bis(2-hydroxyethyl-2-amino ethane sulfonic acid) pH 7.0. The dialysis solutions were prepared with varying concentrations of CaEGTA, but the ratio of total EGTA to total Ca was fixed at 2:1, to give an ionized calcium concentration of 0.2 μM based on an apparent equilibrium constant (Flaschka and Schwarzenbach, 1969) of 5×10^6 M^{-1} for the chelation of Ca by EGTA at pH 7.0. In the [^{14}C]EDTA experiments, the labeled EDTA was present either as the unchelated form, or which various fractions of the EDTA titrated by calcium.

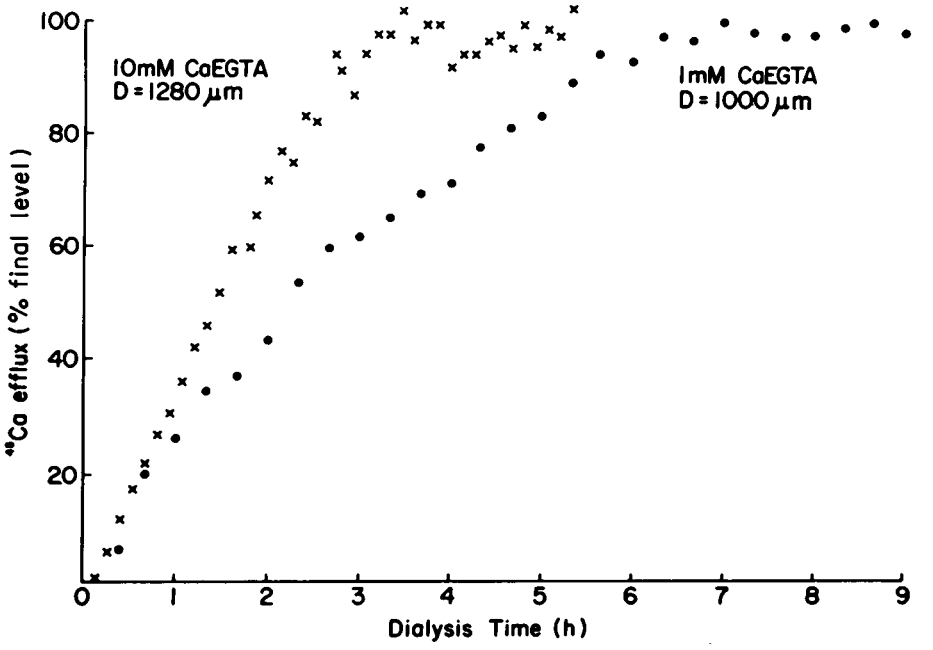

FIGURE 1 Time course of ^{45}Ca efflux during continuous dialysis from two barnacle muscle fibers with the indicated internal CaEGTA concentrations. Efflux expressed relative to the final value.

For calcium analysis, a weighed aliquot of a dialyzed section of fiber was either ashed or digested with concentrated HCl or NaOH, and diluted to a known volume for analysis by atomic absorption spectroscopy. Lanthanum was added to suppress phosphate interference. Another aliquot of the solution was counted to determine the amount of labeled calcium in the fibers. Fibers analyzed for labeled EDTA were first triturated with sand and diluted to a known volume for tracer analysis.

RESULTS

The results of two efflux experiments is shown in Fig. 1. The fluxes have been normalized by expressing them relative to the plateau value. The examples illustrated were selected for comparable diameter so that the diffusion times within the sarcoplasm would be similar. The internal ionized calcium was 0.2 μM. With 10 mM CaEGTA the efflux became constant after 3–4 h of dialysis, but with 1 mM about 5–6 h was required. This is of course to be expected if there were a pool of endogenous calcium in the fiber capable of diluting the specific activity of the labeled calcium in the dialysate. Direct evidence for the existence of such a pool is presented below.

A second point illustrated by the data (summarized in Table I and Fig. 2) is that the plateau level of calcium efflux depends upon the internal concentration of CaEGTA, at a constant ionized calcium concentration. For example the efflux obtained during dialysis with 1 mM CaEGTA is only about 20% of that obtained at the highest con-

TABLE I

CALCIUM CONCENTRATIONS AND EFFLUXES IN DIALYZED BARNACLE MUSCLE FIBERS (15–17°) (INTERNAL IONIZED $C_A = 0.2\ \mu M$)

Experiments	$[CaEGTA]_i$	Fiber diameter	Dialysis time (h:min)	^{45}Ca efflux	Total fiber Ca^{++}	Fiber ^{45}Ca	Unexchanged Ca
	mM	*mm*		*pmol/cm² · s*	*mmol/kg*	*mmol/kg*	*mmol/kg*
101572 no. 1	0.02	1.6	5:12	0.06*	0.85	0.09	0.76
101573 no. 2	0.02	1.2	4:00	0.05*	0.64	0.03	0.61
101673 no. 1	0.02	1.0	6:28	0.06*	—	—	—
101673 no. 2	0.02	1.0	3:14	0.04*	—	—	—
101973	0.02	1.4	8:48	0.04‡	0.44	0.01	0.43
100173	0.1	1.0	4:42	0.12‡	0.50	0.24	0.26
102973	0.1	1.5	5:20	0.09*	0.57	0.06	0.51
012274	0.2	1.2	5:20	0.15*	0.36	0.18	0.18
111373	0.25	0.9	5:20	0.2*	—	—	—
11473	0.25	1.2	5:20	0.11‡	0.70	0.18	0.52
111572	0.25	1.0	7:12	—	0.95	0.29	0.66
110773	0.5	1.2	5:20	0.39*	—	—	—
110873	1	1.2	5:20	0.69*	—	—	—
022574	1	1.4	8:00	0.99	0.89	0.49	0.40
022674 no. 1	1	1.2	8:00	1.27	0.8	0.58	0.22
022674 no. 2	1	1.2	8:00	1.0	—	—	—
030374	1	0.9	9:04	1.05	0.89	0.57	0.32
030674	1	1.3	8:00	1.4	0.73	0.39	0.34
012174	2	0.7	4:08	1.96¶	—	—	—
013074	2	1.2	5:20	1.6	0.85	0.63	(0.22)
013174	2	0.9	5:20	1.83	—	—	—
102574	5	1.0	5:00	3.4‡	2.07	2.02	(0.05)
010274	7.2	1.1	6:40	5.2	—	—	—
121173	9.6	1.2	5:28	5.58§	—	2.17	—
121473	9.6	1.1	5:00	4.6	3.12‖	2.82	(0.3)
121873	9.6	1.0	5:00	3.56¶	2.6‖	2.5	(0.1)
121073	10	1.0	5:36	6.26	—	2.46	—
Mean (*n*)							0.43 (12)

Values in parentheses excluded from the mean.

*Efflux had not reached a steady state.

‡Attainment of a steady-state efflux was uncertain.

¶ T = 10°C.

§External solution contained 10 mM Ca.

‖ Fiber section used for analysis was taken about 5 h after time of reported efflux.

centration used (10 mM). The approach to equilibrium was so slow when fibers were dialyzed with less than 1 mM CaEGTA that it was difficult to obtain a definite plateau before the preparation deteriorated. For that reason, those experiments, represented in Fig. 2 by open circles are actually extrapolated estimates of the plateau value for which no great accuracy is claimed. Nonetheless, a definite dependence upon internal EGTA concentration is clear.

It seems reasonable that the efflux of calcium shown in Fig. 2 and Table I might rep-

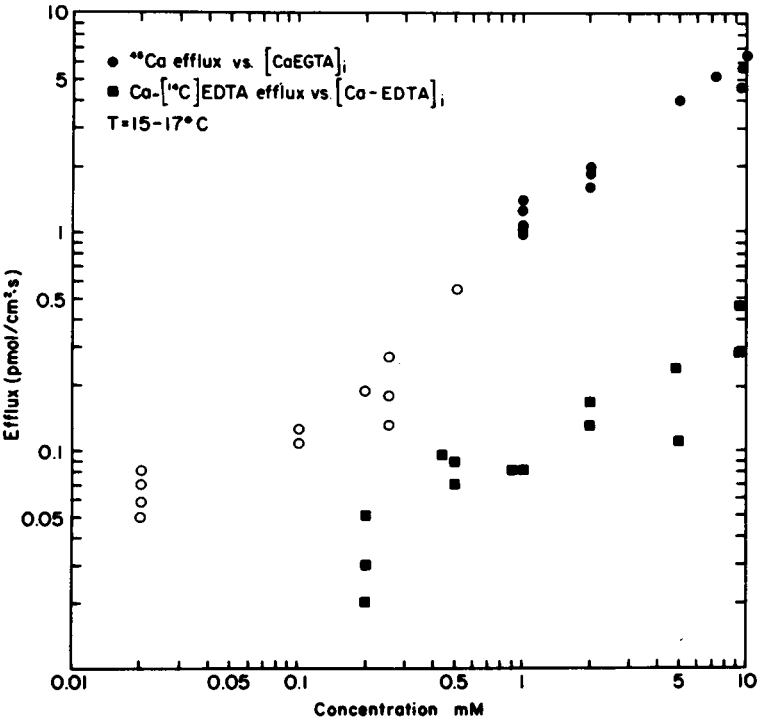

FIGURE 2 ^{45}Ca efflux vs. internal CaEGTA concentration (circles) and [^{14}C]EDTA efflux vs. internal CaEDTA concentration (squares). The open symbols represent experiments in which the plateau was not definitely established.

resent leak of calcium bound to EGTA. The most direct test of this possibility, i.e. comparison of ^{45}Ca efflux with [^{14}C]EGTA efflux was not feasible because carbon labeled EGTA was not commercially available. Instead we used [^{14}C]EDTA for this comparison. Although it is not a priori certain that the permeability of the membrane to EGTA and EDTA are the same, several dialysis experiments using [^{45}Ca]EDTA in place of [^{45}Ca]EGTA gave about the same efflux, therefore, justifying our comparison of [^{45}Ca]EGTA efflux with [^{14}C]EDTA efflux.

The EDTA experiments are also illustrated in Fig. 2, where it can be seen that at all concentrations of EDTA the efflux was small in comparison with the ^{45}Ca efflux, varying from 7 to 12% of the calcium efflux. We infer, therefore, that the leak of calcium bound to EGTA cannot explain the large variation of calcium efflux with CaEGTA concentration.

Another possible interpretation of these results is that most of the ^{45}Ca leaving the fiber is complexed to some other internal anion present in the dialysate.

In the majority of these experiments the principal dialysate anion was aspartate (79 mM). Assuming the equilibrium constant reported by Lumb and Martell (1953) for the binding of calcium by aspartate, simultaneous equilibria calculations for the binding of Ca to aspartate and EGTA predict that the concentration of Ca aspartate is

low (less than 10^{-6} M) and increases (at most) by about 8% while the Ca EGTA concentration ranges from 20 μM (Ca efflux $\approx$ 0.07 pmol/cm^2 · s) to 5 mM (Ca efflux $\approx$ 4 pmol/cm^2 · s). Clearly, it becomes difficult to attribute the Ca efflux primarily to Ca aspartate when its concentration is so low and increases so little while the Ca efflux increases by a factor of about 57.

The present experiments also permitted calculation of the unexchanged calcium, i.e. that calcium which has not been dialyzed out of the fiber. This measurement depends upon subtracting the labeled calcium from the analytical value for total tissue calcium; therefore this estimate is most accurate when the dialysis medium contains little CaEGTA. We have, therefore, rather arbitrarily restricted our measurements to those in which the total CaEGTA was 1 mM or less. The mean value of unexchanged calcium was 0.43 mmol/kg (after 5–8 h of dialysis). The total calcium in fresh barnacle muscle, washed in calcium free solutions, was about 1 mmol/kg. We infer that almost half of the total fiber calcium does not exchange under our conditions.

The calcium efflux from microinjection experiments are calculated on the assumption that the injected calcium has completely exchanged with all of the endogenous fiber calcium. Clearly, if injected fibers respond to the introduction of calcium as do dialyzed fibers, the calculated efflux would be higher than the actual value, the degree of over estimation depending upon the amount of unexchanged endogenous calcium.

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