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Calcium Fluxes in Internally Dialyzed Giant Barnacle Muscle Fibers

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Summary. Calcium-45 fluxes have been examined in isolated giant barnacle muscle fibers subjected to internal solute control by means of "internal dialysis". The ^{45}Ca efflux was dependent upon the concentrations of both total and ionized internal Ca (Ca^{2+} buffered with EGTA). With a total Ca concentration of 2.0 mM and a 1:2 Ca/EGTA ratio (nominal $[Ca^{2+}]_i = 0.13$ μM), the Ca efflux averaged 1.2 pmoles/cm^2 sec. Under identical conditions, the mean Ca influx was only 0.36 pmoles/cm^2 sec. The large Ca efflux may not be attributed to leak of the CaEGTA complex, since a 2.5-fold increase in the EGTA concentration (nominal $[Ca^{2+}]_i = 0.032$ μM) *reduced* the ^{45}Ca efflux by one-third. Furthermore, when EDTA was used to buffer the internal Ca concentration (in the absence of internal Mg), the steady efflux of ^{14}C-EDTA was only about 10% of the ^{45}Ca efflux (in parallel experiments). The time-course of the ^{45}Ca fluxes also appeared anomalous in that ^{45}Ca influx reached a steady level much more rapidly than ^{45}Ca efflux in fibers of comparable diameters. If the muscle fibers are treated as right circular cylinders, these data imply that the apparent diffusion coefficient for inwardly-moving Ca is much larger than for outwardly-moving Ca. In contrast to Ca efflux, the outward diffusion of ^{22}Na, ^{14}C-EDTA and ^3H_2O appears to be limited primarily by the permeability of the dialysis tube wall. Some, but not all, of the anomalous behavior of the Ca fluxes can be reconciled if the deep, branched infoldings of the barnacle muscle surface membrane are taken into account.

There is now considerable evidence that during depolarization an inward calcium current plays an important role in providing Ca for activation of contraction in giant barnacle muscle fibers (Hagiwara, Takahashi & Junge, 1968; Atwater, Rojas & Vergara, 1974). In order for the fibers to remain in steady Ca balance, Ca must be extruded against a large electrochemical gradient during recovery from activity (*cf.* Blaustein, 1974). Calcium efflux from ^{45}Ca-injected barnacle muscle fibers is partially dependent upon external sodium, perhaps indicating that uphill Ca extrusion derives some of its energy from the downhill movement of Na,

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via a Na–Ca counterflow exchange mechanism (Russell & Blaustein, 1974). For a more detailed examination of the influence of ion electrochemical gradients and intracellular energy stores on Ca efflux, a method of internal solute control is required. The internal dialysis technique (Brinley & Mullins, 1967) would appear to be well-suited to this problem. The dialysis technique has been adapted for the study of univalent ion fluxes in barnacle muscle (DiPolo, 1972; DiPolo & LaTorre, 1972), and has recently been used successfully for Ca influx measurements in this preparation (DiPolo, 1973).

In the present study the internal dialysis technique has been employed to investigate the effects of internal total Ca (as CaEGTA[1]) and free, ionized Ca ($[Ca^{2+}]_i$) on ^{45}Ca efflux from barnacle muscle fibers. The results indicate that the concentrations of both forms of Ca influence the Ca efflux. However, in parallel experiments with ^{45}CaEDTA[2] and Ca(^{14}C-)EDTA, the efflux of ^{14}C was less than 10 % of the ^{45}Ca efflux. Similar observations have recently been reported by Brinley and Spangler (1975).

Influx of ^{45}Ca was also determined in several muscle fibers. The influx rose to a steady level much more rapidly than did the Ca efflux; however, the steady influx was only about one-third to one-fourth of the steady efflux.

The results are discussed in terms of the barnacle muscle fiber morphology. We conclude that some of the anomalous flux behavior may be a consequence of the deep, branched invaginations of the surface membrane (Hoyle, McNeill & Selverston, 1973).

A preliminary account of these observations has been communicated to the Biophysical Society (Russell & Blaustein, 1975).

Materials and Methods

Large specimens of *Balanus nubilus* or *Balanus aquila* (from Pacific Biomarine Supply Co., Venice, Calif.) were dissected to obtain groups of muscle fibers from either the depressor scutorum rostralis or lateralis with their insertions onto small chunks of basal plate still intact. Single fibers were separated by cutting their tendons free of scutal connective tissue and carefully cutting and tearing the strands of connective tissue between the fibers with fine scissors and glass needles. Fibers not immediately used were stored in barnacle sea water at 12 °C for use the following day. As indicated previously (Russell & Blaustein, 1974), intact fibers appeared to remain in good physiological condition for at least 24–36 hr.

1 EGTA = ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetate.
2 EDTA = ethylenediamine tetraacetate.

Solutions

External Solutions. The composition of the barnacle sea water (BSW) was similar to that used previously (Russell & Blaustein, 1974) and its composition was (in mmoles/liter): 440 NaCl, 10 KCl, 11 $CaCl_2$, 32 $MgCl_2$, 5.2 Tris, 5.0 HEPES, pH 7.9. Calcium-free barnacle sea water had $CaCl_2$ replaced mole-for-mole with $MgCl_2$. The osmolalities of these solutions were routinely checked by means of a freezing point depression osmometer and were in the range of 955 ± 5 milliosmoles/kg.

Internal Dialysis Solution. The standard dialysis solution contained (in mmoles/liter): 142 K isethionate, 38 KCl, 5 NaCl, 10 HEPES, 2.9 Tris, 431 sucrose, 0.5 phenol red, pH 7.1; osmolality 930 ± 5 milliosmoles/kg. The dialysis solution also contained $CaCl_2$ and a Ca-buffering ligand (EGTA or EDTA); the concentrations of these agents will be indicated for each experiment (*see* Results). The calculated "nominal" concentrations of Ca in the EGTA- and EDTA-buffered solutions were based on a stability constant of 7.6×10^6 M^{-1} for CaEGTA and 3.1×10^7 M^{-1} for Mg-free CaEDTA (*cf.* Portzehl, Caldwell & Rüegg, 1964). The contamination by Ca (determined by atomic absorption spectroscopy) of K isethionate (148 μmoles per mole) and of sucrose (27 μmoles per mole) as well as the contribution of the Ca from the $^{45}CaCl_2$ were all taken into account when calculating total Ca concentration. The concentrations of the major cations in the dialysis solution were based upon the analyses of a large number of muscle fibers (Blaustein, *unpublished data*). The HCl solution containing the radionuclide was evaporated to dryness and re-dissolved in a Tris-HEPES buffer solution of pH 7.1 to a convenient level of radioactivity before aliquots were added to the dialysis fluids or BSW.

Dialysis Procedure

Dialysis Capillaries. Cellulose acetate tubing, supplied by Dr. E. Ashare of Fabric Research Laboratories, Inc., Dedham, Mass., was used to prepare porous dialysis capillaries. This tubing had an outer diameter of 195 μ and an inner diameter of 145 μ. The central 16–18 mm of a 10–14 cm length of tubing was hydrolyzed in 0.1 N NaOH for 15–20 hr in order to render it porous. The tubing was then mounted in a lucite T-block (*see* Fig. 1) which directed fluid flow through the hollow center of the cellulose acetate tubing. The dialysis methods employed were essentially those of Brinley and Mullins (1967) including their recent modifications (Brinley & Mullins 1974).

Dialysis Chamber. The chamber (Fig. 1) was similar to the one originally designed by Brinley and Mullins (1967) for use with squid axons.

Experimental Procedures. Separated single fibers, still attached to the baseplate were allowed to remain in Ca-containing seawater at 12 °C for at least one hour following dissection, Fibers damaged during dissection were detected by the appearance of gross or localized contractions and were discarded. A group of intact, undamaged fibers was then placed in Ca-free barnacle sea water which was changed 2–4 times over a 30–40 min period. A single fiber was cut as close as possible to its insertion on the base plate. The fiber was then transferred to the dialysis chamber which also contained Ca-free sea water. The ends of the fiber were cannulated using thin-walled glass capillaries of about 400 μ diameter. These "end-cannulae" were filled with a solution containing 275 mM EGTA-75 mM $MgSO_4$, pH 7.1, to avoid contractures at the cut ends of the fiber.

The dialysis tubing, stiffened by an axial 3–4 mil tungsten wire, was threaded through the muscle fiber from right to left under microscopic examination, until the middle of the porous region (1.8 cm long) was centered in the collection chamber (compartment *B* in Fig. 1) which was 1.3 cm wide. The stiffening wire was then removed. In order to measure membrane potential a glass capillary, approximately 100 μ in diameter, filled with 3 M KCl, was guided

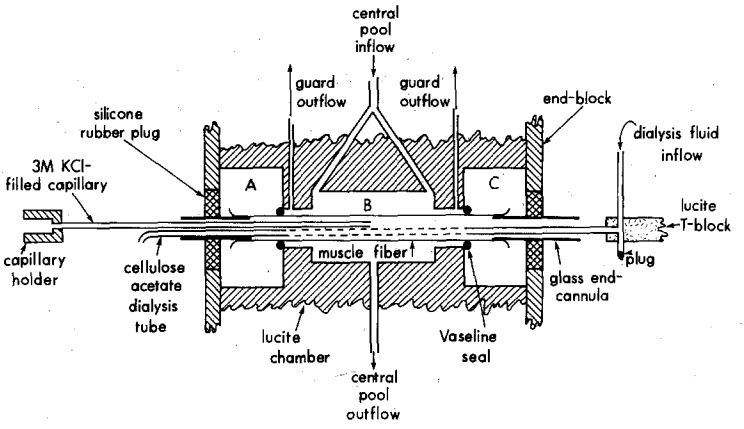


Fig. 1. Diagrammatic view of the experimental chamber. The view represents a cross-section through the chamber, parallel to the upper surface. It is not drawn to scale, and is meant primarily to indicate the relative positions of the muscle fiber, hollow cellulose acetate fiber, chamber compartments, and internal electrode. The membrane potential was recorded via two calomel half-cells. One electrode was in contact with the KCl-filled capillary; the "indifferent" calomel cell made contact (through a KCl bridge) with the fluid flowing into the central pool. The narrow regions of the chamber between compartment *B* and compartments *A* and *C* are the "guard" regions – i.e., the regions surrounding the lateral reaches of the dialyzed portion of the muscle fiber. About 3 % (or 0.04 ml/min) of the fluid entering the central pool superfused these portions of the muscle fiber and exited through the guard outflows. Only the fluid leaving via the central pool outflow (1.4 ml/min) was sampled for tracer content during efflux experiments. For ^{45}Ca influx measurements, the central pool inflow and outflow ports were sealed off. Isotope-labeled BSW was then circulated through compartment *B* by slowly flowing the solution into one guard outflow, and out the other guard outflow, via a push-pull syringe arrangement. Brinley and Mullins (1967) should be consulted for details of chamber design

through the left-hand end-cannula and into the muscle until its tip was in the middle of the dialyzed portion of the muscle fiber. Greased blocks and Vaseline seals (*see* Fig. 1 and Brinley & Mullins, 1967) were used to isolate the region to be dialyzed from the cut ends of the fiber. The flow of dialysis fluid through the cellulose acetate fiber was then initiated and the BSW was perfused through compartment *B* around the muscle fiber. The cut ends remained in Ca-free BSW (in compartments *A* and *C* of Fig. 1), with EGTA added to prevent contractures.

Efflux Experiments

Effluxes were measured by adding radioisotope to the dialysis fluid and collecting and counting the external, superfusing fluid. The dialysis fluid was usually delivered at the rate of 1 μliter/min from a motor-driven syringe pump. In several experiments the flow rate was increased to 6 μliter/min with no noticeable difference in results. The external solution flowed over the fiber at the rate of 1.4 ml/min from a pair of peristaltic pumps and was collected by another peristaltic pump. The temperature in the chamber was controlled at 15 ± 1 °C, and was monitored with a thermistor in the central pool just below the muscle fiber.

Influx Experiments

The same chamber was used for both efflux and influx experiments. In the latter case, however, the central pool inflow and outflow were closed off. The radioisotope-containing solution (BSW) was delivered from a pair of push-pull syringes, via the guard ports, the direction of fluid flow being reversed every 20 min; the flow rate was 0.4 ml/min. Radioactivity appearing in the dialysis fluid was collected from the open tip of the dialysis tube. Temperature was maintained at 15 ± 1 °C.

Counting Procedures

Efflux. Aliquots of timed samples of the superfusing sea water were placed in scintillation vials and 14 ml of Bray's cocktail (Bray, 1960) or a toluene-Triton X-100 cocktail (Nadarajah, Leese & Joplin, 1969) was added. The samples were counted in a Packard Tricarb liquid scintillation counter.

Samples labeled with only ^{45}Ca or ^{14}C-EDTA could be counted in a straightforward way. In several experiments, however, in addition to ^{45}Ca, ^{22}Na and tritiated water were present in the fluid to be counted. One aliquot was counted in a Packard Auto-gamma counter to determine ^{22}Na activity. Another aliquot was counted in the scintillation counter with one window set for optimal detection of ^{3}H activity and one for detection of ^{45}Ca activity. It was determined by carefully prepared standards that 100 % of the ^{22}Na counts appeared in the ^{45}Ca window whereas only 2 % appeared in the ^{3}H window. Thirteen percent of the ^{45}Ca counts spilled into the tritium window; a negligible fraction of the tritium appeared in the ^{45}Ca window. Thus, appropriate corrections could be made which permitted the simultaneous counting of all three isotopes.

Influx. Timed samples were collected directly from the dialysis tube tip into scintillation vials; 2.8 ml of BSW and 14 ml of scintillation cocktail were added. The counting procedures were similar to those used for efflux samples.

All isotope fluxes will be given in terms of pmoles/cm^2 sec. The surface area calculation assumes that the muscle fibers are cylindrical; the surface area contributed by the deep sarcolemmal invaginations and by transverse tubules has been ignored. Thus, if the fluxes discussed here take place across the entire sarcolemmal surface, the values given below (in terms of surface area) may be more than 20-fold too large (*cf.* Hoyle *et al.*, 1973).

Results

Membrane Potential

The membrane potential of a fiber immediately after it had been prepared for dialysis was usually in the range of -40 to -45 mV. As soon as Ca-containing BSW was re-introduced into the central compartment, and the flow of dialysis fluid begun, the membrane potential would begin to hyperpolarize slowly. A steady resting potential was usually attained within 90 min. The potential then generally remained constant for 4–6 hr, and occasionally for as long as 10 or 12 hr, when the fibers were bathed in standard BSW. Since the fibers were dialyzed with tracer-free solution for 40–70 min before isotope was added, most of the membrane hyper-

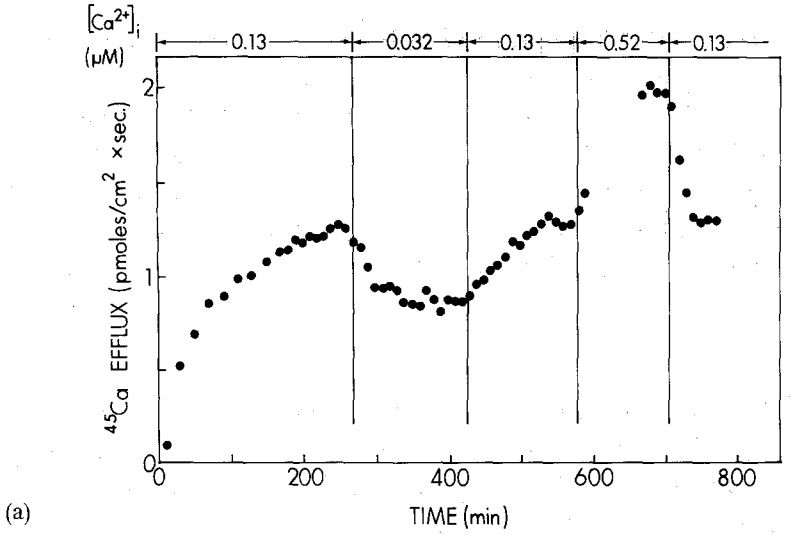
polarization occurred before isotopic flux measurements were begun. The average steady resting potential was −50 mV (*cf.* Tables 1 and 3) which is quite close to the −53 mV value reported by DiPolo and LaTorre (1972) and −58 mV reported by DiPolo (1972). The difference between their values and ours may be due to the fact that our experiments were conducted at 15 °C, rather than 22 °C, and the lower temperature would be expected to depolarize the membrane (DiPolo & LaTorre, 1972).

^{45}Ca *Efflux*

When ^{45}Ca was added to the dialysis fluid, tracer began to appear in the superfusing BSW within 2–4 min. However, the approach to a steady level of ^{45}Ca efflux was very slow, and in several early experiments internal or external solution changes were made before a steady efflux had been attained. Once the long time-course for ^{45}Ca efflux buildup became evident (e.g. *see* Fig. 2*A*), we usually waited until a steady efflux was reached before changing the experimental conditions. The data from these experiments are summarized in Table 1. It took from 3–5 hr, and sometimes longer, to reach a steady efflux (the rise-time, τ, for ^{45}Ca efflux is given in column 8 of Table 1). This is somewhat slower than the time required for injected fibers to reach their steady-state rate constants for efflux (1 to 2 hr; Ashley, Caldwell & Lowe, 1972; Russell & Blaustein, 1974) and much slower than the time required for monovalent ions to reach steady efflux levels in dialyzed barnacle preparations (DiPolo, 1972; DiPolo & LaTorre, 1972; and *see below*). Furthermore, DiPolo (1973) reports (and *see below*) that ^{45}Ca influx in dialyzed fibers attained steady levels within 30–60 min of beginning isotopic flux measurements. The problem of the slow time-course of ^{45}Ca efflux will be reconsidered in a subsequent section.

With a total Ca concentration of 2.0 mM, and an EGTA concentration of 4.0 mM in the dialysis fluid, giving a nominal $[Ca^{2+}]_i$ of 0.13 μM, Ca efflux averaged 1.2 ± 0.3 pmoles/cm^2 sec (mean ± SE; *see* Table 1). This value agrees well with data obtained from ^{45}Ca-injected giant barnacle muscle fibers if most of the Ca in the sarcoplasm is exchangeable (Ashley *et al.*, 1972; Russell & Blaustein, 1974).

Not unexpectedly, the Ca efflux was very much dependent upon the concentration of ionized Ca, $[Ca^{2+}]_i$, in the dialysis fluid. This is clearly illustrated by the experiment of Fig. 2*A* (*also see* the data for fibers 07313 and 08013 in Table 1), in which the total Ca concentration was maintained constant and $[Ca^{2+}]_i$ was altered by varying the EGTA concentration. The effluxes from this experiment are regraphed as a function of $[Ca^{2+}]_i$



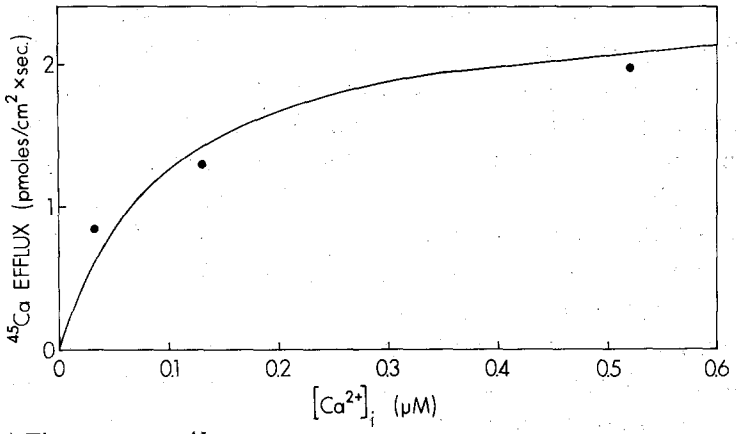


Fig. 2. (a) Time-course of ^{45}Ca efflux from a giant barnacle muscle fiber, and the effect of $[Ca^{2+}]_i$ on Ca efflux. At the beginning of the experiment the fiber was dialyzed with a solution containing 2.0 mM Ca and 4.0 mM EGTA (nominal $[Ca^{2+}]_i = 0.13$ μM). The Ca concentration was maintained at 2.0 mM while, during the periods indicated on the graph, the EGTA concentration was reduced to 2.5 mM (nominal $[Ca^{2+}]_i = 0.52$ μM) or increased to 10 mM (nominal $[Ca^{2+}]_i = 0.032$ μM). The original dialysis fluid was re-introduced in the middle of the experiment, and again at the end. Isotope was first introduced at zero-time. Fiber 11273. Diameter, 1.39 mm. Resting potential, −47 mV. Temperature, 15.5 °C. (b) Ca efflux graphed as a function of $[Ca^{2+}]_i$. The data are from the same experiment as those of Fig. 2(a). The curve has been drawn to fit the equation:

$$M_0^{Ca} = \frac{\overset{*}{M}_0^{Ca}}{1 + \frac{K_{Ca}}{[Ca^{2+}]_i}}$$

where $\overset{*}{M}_0^{Ca}$ is the maximal Ca efflux, with a value of 2.5 pmoles/cm^2 sec, and M_0^{Ca} is the efflux at any $[Ca^{2+}]_i$. The values of $[Ca^{2+}]_i$ were based on a CaEGTA stability constant of 7.6×10^6 M^{-1} (Portzehl *et al.*, 1964). The apparent half-saturation constant for Ca^{2+}, K_{Ca}, had a value of 0.1 μM. A slightly better fit to the equation is obtained if the fluxes are "corrected" for a CaEGTA "leak" flux of about 0.1 pmoles/cm^2 sec (*see text*)

Table 1. Steady ^{45}Ca efflux from dialyzed barnacle muscle fibers into standard barnacle sea water

Fiber	Total $[Ca]_i$	$[EGTA]_i$	Nominal $[Ca^{2+}]_i$[a]	^{45}Ca Efflux	Membrane potential	Fiber diameter	τ[b]	D'_{Ca}[c]
	(mM)	(mM)	(μM)	(pmoles/ $cm^2 \times sec$)	(mV)	(mm)	(min)	($\times 10^6$ cm^2/sec)
07113	2.0	4.0	0.13	1.55	–	1.55	40	2.13
07123	2.0	4.0	0.13	1.42	–	1.25	60	0.81
07243	2.0	4.0	0.13	0.76	−48	1.36	72	0.85
08303	2.0	4.0	0.13	1.73	−58	0.98	115	0.22
10093a	2.0	4.0	0.13	0.89	−53	1.10	115	0.30
10093b	2.0	4.0	0.13	0.77	−50	1.38	180	0.35
10153	2.0	4.0	0.13	1.63	−50	1.48	115	0.66
10173	2.0	4.0	0.13	0.83	−51	1.60	225	0.41
10243	2.0	4.0	0.13	1.2	−50	1.38	180	0.35
11273	2.0	4.0	0.13	1.25	−47	1.39	80	0.81
Mean				1.20				0.69
09203	0.058	0.109	0.13	0.07	−48	1.75	140	0.83
10033	0.058	0.109	0.13	0.06	−47	1.10	160	0.22
10043	0.058	0.109	0.13	0.04	−54	1.30	180	0.30
Mean				0.06				0.45
09193	0.054	0.70	0.011	0.025	−56	1.50	150	0.52
11273	2.0	10.	0.032	0.84	−47	1.39	–	–
08013	2.0	6.34	0.064	1.07	−42	0.83	36	0.45
11273	2.0	2.5	0.52	1.98	−47	1.39	–	–
07313	2.0	2.5	0.52	1.60	−35	0.88	75	0.25

[a] Based on a CaEGTA stability constant of 7.6×10^6 M^{-1} (Portzehl *et al.*, 1964).
[b] Rise-time (τ) is time required for flux to reach 63 % of its steady value.
[c] Apparent Ca diffusion coefficient; *see* Appendix, Eq. (7).

in Fig. 2*B*. The data suggest that Ca efflux saturates with increasing $[Ca^{2+}]_i$; the apparent half-saturation constant (K_{Ca}) is about 0.1 μM Ca.

A rather surprising observation was that ^{45}Ca efflux depended upon the total Ca concentration, when $[Ca^{2+}]_i$ was maintained constant with a fixed Ca/EGTA ratio. The data in Table 1 show that when CaEGTA was increased from 0.06 to 2.0 mM, Ca efflux increased, on the average, from 0.06 to 1.2 pmoles/cm^2 sec. Vogel and Brinley (1973) observed a ^{45}Ca efflux of 0.5 pmoles/cm^2 sec when they dialyzed barnacle muscle fibers with a solution containing 0.33 mM CaEGTA and a nominal $[Ca^{2+}]_i$ of 0.25 μM. Their Ca efflux value is certainly in good agreement with the data in Table 1, given the differences in $[CaEGTA]_i$ and $[Ca^{2+}]_i$.

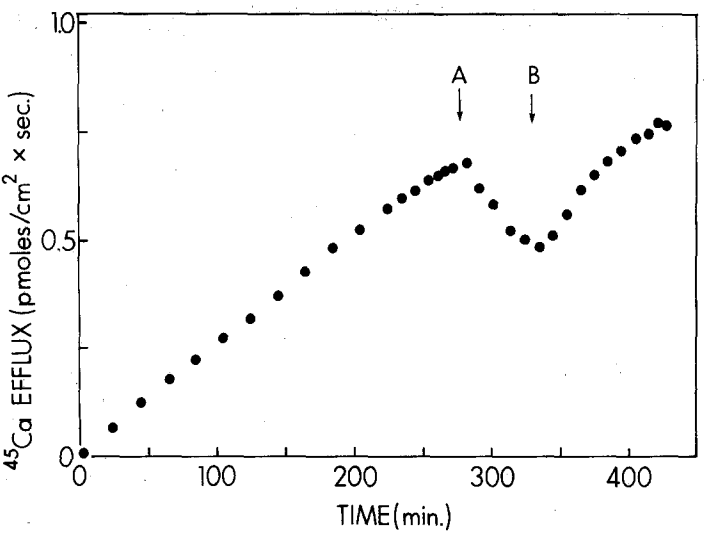


Fig. 3. Dependance of ^{45}Ca efflux upon the total calcium concentration in the dialysis fluid. The fiber was initially dialyzed with fluid containing 2.0 mM Ca and 4.0 mM EGTA. At *A* this dialysis fluid was replaced by one containing 40 μM Ca and 80 μM EGTA, so that the nominal $[Ca^{2+}]_i$ remained constant at 0.13 μM. The original dialysis fluid was re-introduced at *B*. Fiber 10093b. Diameter, 1.38 mm. Resting potential, −50 mV. Temperature, 15 °C

In most cases $[CaEGTA]_i$ was maintained constant throughout the experiment. However, in one instance the fiber was first dialyzed with a high CaEGTA concentration (2.0 mM), and then both the total $[Ca]_i$ and $[EGTA]_i$ were reduced so that the nominal $[Ca^{2+}]_i$ remained constant. The Ca efflux declined from about 0.7 to about 0.5 pmoles/cm^2 sec (Fig. 3); this decrease is much less than would be expected from the 40-fold decrease in $[CaEGTA]_i$ if Ca efflux is indeed nearly proportional to $[CaEGTA]_i$. One possibility is that we did not wait until a steady efflux was reached before changing $[CaEGTA]_i$. However, the changes in efflux appeared to occur much more rapidly after the fiber was loaded with CaEGTA *(see below)*; this may indicate that a slowly loaded compartment within the sarcoplasm contributes to the anomalous behavior.

Efflux of ^{14}C-EDTA

The observation that Ca efflux is significantly influenced by the concentration of CaEGTA raises the possibility that much of the Ca loss may be due to efflux of the CaEGTA complex. This possibility seems unlikely because Ca efflux is also very much dependent upon $[Ca^{2+}]_i$, and at a constant total Ca concentration (i.e., virtually constant

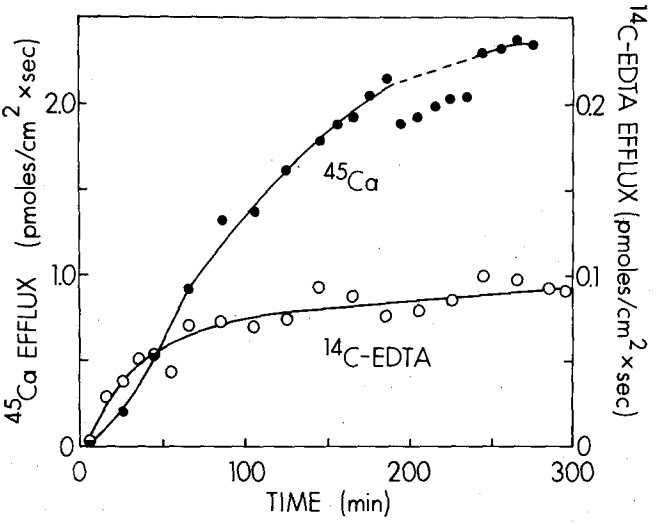


Fig. 4. Efflux of ^{14}C-EDTA (○) and ^{45}Ca (●) from a pair of barnacle muscle fibers. Both fibers were dialyzed with Mg-free solution containing 2.0 mM Ca and 2.5 mM EDTA (nominal $[Ca^{2+}]_i = 0.13$ μM; based on a CaEDTA stability constant of 1.1×10^7 M^{-1}). In one case (fiber 12183; diameter, 1.71 mm; resting potential, −47 mV; temperature, 15.5 °C), the dialysis fluid was labeled with ^{14}C-EDTA. Another fiber from the same barnacle (# 12193; diameter 1.10 mm; resting potential, −47 mV; temperature, 15 °C) was dialyzed with ^{45}Ca-labeled fluid. Note the 10-fold difference in the ordinate scales

$[CaEGTA]_i$), Ca efflux is increased by lowering $[EGTA]_i$ and decreased by raising $[EGTA]_i$ (Fig. 2). Nevertheless, more direct information about possible leak of the CaEGTA complex would be welcome. Since isotopically-labeled EGTA is not readily available, ^{14}C-EDTA was used to directly determine the rate of CaEDTA efflux. Fig. 4 shows data from a pair of experiments in which the Ca in the dialysis fluid was buffered with EDTA; $MgCl_2$ was omitted from the solution so that $[Ca^{2+}]_i$ could be buffered at about the same level as that usually maintained with EGTA. Taken at face value, these results, and the data from one other experiment with ^{14}C-EDTA (efflux = 0.25 pmoles/cm^2 sec), indicate that only a small fraction (less than 10 %) of the Ca efflux can be accounted for by efflux of the chelate complex (but *see* Discussion).

Simultaneous Efflux of ^{22}Na, Tritiated Water and ^{45}Ca

The long rise-time for the ^{45}Ca efflux, mentioned above, is somewhat disconcerting in view of the limits of dialyzed muscle fiber viability – only 5–7 hr, in many instances. In order to see whether or not this long

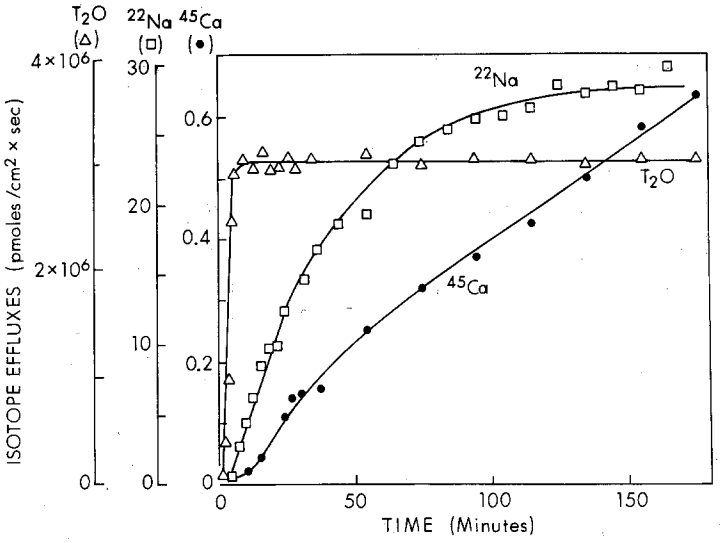


Fig. 5. Simultaneous efflux of ^{45}Ca (●) ^{22}Na (□) and tritiated water (T_2O; △) into barnacle sea water. In this experiment the dialysis fluid contained 16 mM NaCl, 2.0 mM Ca and 4.0 mM EGTA. Isotopically-labeled dialysis fluid was introduced at zero-time, but the abscissa time scale does not reflect a 60-sec dead-time between the chamber and the collection vial. Fiber 10233. Resting potential, −46 mV. Diameter, 1.40 mm. Temperature 14.5 °C

rise-time was peculiar to Ca efflux, the fluxes of ^{22}Na, tritiated water (T_2O) and ^{45}Ca were directly compared. Fig. 5 gives the results of one of two triple-label experiments.

The steady Na efflux into BSW when $[Na]_i$ was 16 mM, 29 pmoles/cm² sec, for the experiment of Fig. 5, with a mean of 23 pmoles/cm² sec for three experiments, may be compared with data obtained by Brinley (1968). In intact, ^{22}Na-injected fibers, he obtained a mean Na efflux of 39 pmoles/cm² in fibers with an average Na concentration of 19 mmoles/kg fiber wet weight (or 27 mmoles/kg intracellular water; *cf.* Gayton, Allen & Hinke, 1969). In four fibers dialyzed with fluid containing 15 mM Na, Brinley (1969) measured a mean Na efflux of 35 pmoles/cm² sec.

In a fiber (10153 of Table 2) dialyzed with the standard fluid containing only 5 mM Na, the ^{22}Na efflux was 6 pmoles/cm² sec. However, even in this case, the rise-time for ^{22}Na efflux was not very different from that obtained in fibers dialyzed with 15 mM Na. Table 2 lists the rise-times and the "apparent diffusion coefficients" (D') for diffusion of the ^{22}Na through the cellulose acetate fiber wall plus sarcoplasm (*see* Appendix). The mean apparent diffusion coefficient, 2×10^{-6} cm²/sec, is about half the diffusion coefficient for Na in barnacle muscle sarcoplasm obtained by Caillé and Hinke (1972), 4.38×10^{-6} cm²/sec. Our low value may indicate that the

Table 2. Rise-time and diffusion coefficient data for ^{22}Na, T_2O, EGTA and ^{14}C-EDTA

	Fiber	Fiber diameter (mm)	τ[a] (min)	D'[b] ($\times 10^6$ cm^2/sec)	Type of measurement[c]
^{22}Na	10153	1.48	29	2.61	^{22}Na efflux
	10173	1.60	49	1.89	^{22}Na efflux
	10233	1.40	40	1.64	^{22}Na efflux
	10243	1.38	37	1.71	^{22}Na efflux
			Mean	1.96	
T_2O	10233	1.40	4	16.42	T_2O efflux
	10243	1.38	4	15.83	T_2O efflux
			Mean	16.13	
EGTA	10093	1.38	30.5	2.08	Δ ^{45}Ca efflux (2 → 0.04 mM Ca EGTA)
			39	1.62	Δ ^{45}Ca efflux (0.04 → 2 mM CaEGTA)
			Mean	1.85	
	11273	1.40	34	1.93	Δ ^{45}Ca efflux (4 → 10 mM EGTA)
			56	1.17	Δ ^{45}Ca efflux (10 → 4 mM EGTA)
			18	3.65	Δ ^{45}Ca efflux (2.5 → 4 mM EGTA)
			Mean	2.25	
^{14}C-EDTA	12043	1.70	40	2.70	^{14}C-EDTA efflux
	12183	1.72	56	1.99	^{14}C-EDTA efflux
			Mean	2.35	

[a] Rise-time (τ) is time required for flux to reach 63 % of its steady value, after introducing isotope, or changing EGTA or CaEGTA concentration.

[b] Apparent diffusion coefficient; *see* Appendix, Eq. (7).

[c] The rate of EGTA diffusion was determined by measuring the rise-time for the change (Δ) in ^{45}Ca efflux when $[EGTA]_i$ (fiber 11273) or $[CaEGTA]_i$ (fiber 10093b) was altered (*cf.* Figs. 2*A* and 3).

rate-limiting factor affecting the time-course of Na efflux from dialyzed fibers is the diffusion of Na through the dialysis tube wall. Indeed, Brinley and Mullins (1974) have obtained a diffusion coefficient of about 2×10^{-6} cm^2/sec for Na diffusion through the porous wall of hollow cellulose acetate fibers.

The apparent diffusion coefficient for T_2O (16×10^{-6} cm^2/sec; Table 2), likewise probably reflects some delay in diffusion through the cellulose acetate fiber wall, since this value is about two-thirds of the value obtained for water diffusion in barnacle muscle sarcoplasm (24.2×10^{-6} cm^2/sec; Bunch & Kallsen, 1969).

The behavior of ^{45}Ca differs considerably from T_2O and ^{22}Na. As noted above, the rise-time for ^{45}Ca efflux is generally quite long. The calculated diffusion coefficient (D'_{Ca}; Table 1) averaged only about one-third to one-fourth of the calculated diffusion coefficient for Na (Table 2), whereas the diffusion coefficients for Ca and for CaEGTA in the porous cellulose tube wall are about half the diffusion coefficient for Na (Brinley & Mullins, 1974). This may indicate that in the case of Ca, diffusion through the sarcoplasm is rate-limiting. On the face of it, this may not seem surprising because the diffusion coefficient for Ca in sarcoplasm is known to be very small (0.14×10^{-6} cm^2/sec in frog muscle; Kushmerick & Podolsky, 1969) due to binding to intrasarcoplasmic structures (e.g., Costantin, Franzini-Armstrong & Podolsky, 1965). In squid nerve axoplasm, too (Hodgkin & Keynes, 1957; and *see* Blaustein & Hodgkin, 1969), the Ca diffusion coefficient is only about 1/50th of the value in free solution (about 8×10^{-6} cm^2/sec; Wang, 1953). However, these low Ca diffusion coefficients refer to free Ca^{2+}, whereas in most of our experiments nearly all of the Ca was added as CaEGTA. [In one fiber, which was dialyzed with an EGTA-free solution containing 8.6 μM Ca (total), the Ca efflux rose steadily, and showed no sign of leveling off when the experiment was terminated after 500 min. The τ was >260 min, and D'_{Ca} was calculated to be $<0.40 \times 10^{-6}$ cm^2/sec in this 1.67 mm diameter fiber.]

The diffusion coefficients for Ca, CaEGTA and EDTA in the wall of the porous cellulose acetate fiber are all about 1×10^{-6} cm^2/sec (Brinley & Mullins, 1974). The apparent diffusion coefficients for the ligands in dialyzed muscle fibers were obtained by measuring the time course of ^{14}C-EDTA efflux (e.g., Fig. 4) or the time-course of the change in Ca efflux when the EGTA or CaEGTA concentration in the dialysis fluid was altered (Figs. 2*A* and 3). The results are summarized in Table 2; they show that D' for EDTA and EGTA averaged about 2×10^{-6} cm^2/sec, indicating that the ligands diffuse through sarcoplasm about as readily as through the porous cellulose acetate fiber wall.

The conclusion from these data is that diffusion of Ca, but not ligand, through barnacle muscle sarcoplasm is retarded (presumably because of Ca binding), even if nearly all of the Ca is introduced in the form of CaEGTA or CaEDTA.

In one preliminary experiment, 5 mM caffeine was added to the sea water when the flow of ^{45}Ca-labeled dialysis fluid was started. Although it was hoped that this treatment would significantly reduce possible Ca binding (in sarcoplasmic reticulum—*cf.* Ashley *et al.*, 1972; Chen, Bittar, Tong & Danielson, 1972) and shorten the Ca equilibration time, the rise-time for

^{45}Ca efflux in this 1.10 mm diameter fiber was 46 min, which corresponds to a D'_{Ca} of 0.76×10^{-6} cm^2/sec.

^{45}Ca Influx

Two interesting observations emerge from a comparison of DiPolo's (1973) barnacle muscle Ca influx data with the Ca efflux data described above. In the first place, ^{45}Ca influx apparently rises to a steady level much more rapidly than does Ca efflux (Table 3 shows values for D'_{Ca} based on DiPolo's influx data and assumed fiber diameters since the diameters are not mentioned in the original report). A second important point is that the Ca influx from sea water was only 0.25 pmoles/cm^2 sec – nearly fivefold lower than the Ca efflux from fibers dialyzed with 2.0 mM Ca. The conditions employed by DiPolo (25 mM Ca in the sea water, and 7.5 mM EGTA-Ca buffer in the dialysis fluid with a 1:7 Ca/EGTA ratio to give a nominal $[Ca^{2+}]_i$ of 5×10^{-8} M) were somewhat different from those used in the present series of efflux experiments. We therefore carried

Table 3. Steady Ca influx in dialyzed barnacle muscle fibers

Fiber	Total $[Ca]_i$ (mM)	$[EGTA]_i$ (mM)	Nominal $[Ca^{2+}]_i$[a] (μM)	^{45}Ca Influx (pmoles/cm^2/sec)	Membrane potential (mV)	Fiber diameter (mm)	τ[b] (min)	D'_{Ca}[c] ($\times 10^6$ cm^2/sec)
12043	2.0	4.0	0.13	0.43	−47	1.65	14	7.14
01034	2.0	4.0	0.13	0.34	−52	1.60	22	4.20
01044	2.0	4.0	0.13	0.32	−54	1.63	45	2.16
Mean				0.36				4.50
01044	2.0	2.5	0.52	0.32	−54	1.63	–	
DiPolo (1973) Fig. 1	1.07	7.5	0.05	[d]	–	1.10[e] / 1.50	25	1.39 / 3.14
DiPolo (1973) Fig. 2	1.07	7.5	0.05	[d]	–	1.10[e] / 1.50	10.5	3.32 / 7.48

[a] Based on a CaEGTA stability constant of 7.6×10^6 M^{-1} (Portzehl *et al.*, 1964).
[b] Rise-time (τ) is time required for flux to reach 63 % of its steady value.
[c] Apparent Ca diffusion coefficient; *see* Appendix, Eq. (7).
[d] Ca influx values for DiPolo's (1973) experiments are not included here because they were obtained with either a high internal (Fig. 1) or low external (Fig. 2) Na.
[e] Assumed values of fiber diameters were used to calculate D'_{Ca} for the experiments of DiPolo (1973) since these data are not given in the original article.

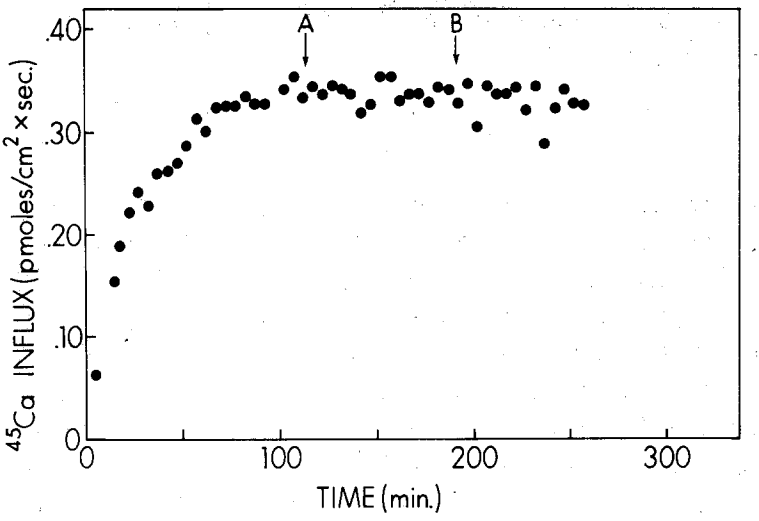


Fig. 6. Calcium influx from standard barnacle seawater. The fiber was dialyzed with fluid containing 2.0 mM Ca and 4.0 mM EGTA. Oligomycin (25 μg/ml) and NaCN (2.0 mM) were added to the dialysis solution at *A*. At *B*, caffeine (2.0 mM) was included in the dialysis fluid along with the oligomycin and cyanide. The pH of the dialysis fluid was readjusted to 7.1 after each addition. Isotope was first introduced into external medium at zero-time. Fiber 01034. Resting potential, −52 mV. Diameter, 1.60 mm. Temperature 14.5 °C

out three ^{45}Ca influx experiments under conditions identical to those used for many of our ^{45}Ca efflux experiments (including the 40–70 min isotope-free pre-dialysis period). Data from one influx experiment are illustrated in Fig. 6, and the results of all three experiments are summarized in Table 3. These data confirm DiPolo's observations: it is clear that under the conditions of these experiments the magnitude of the Ca efflux is significantly greater than the magnitude of the Ca influx, and D'_{Ca} for influx is greater than for efflux (*cf.* Table 1).

In dialyzed squid axons, by way of contrast, rather comparable values for D'_{Ca} were obtained from ^{45}Ca influx and from efflux measurements (Blaustein and Russell, *unpublished data*), using the method given in the Appendix: $0.12 \pm 0.01 \times 10^{-6}$ cm^2/sec (mean ± SE, $n=4$), and $0.26 \pm 0.07 \times 10^{-6}$ cm^2/sec ($n=6$), respectively. Furthermore, the rise-time found upon solving the diffusion equation for a hollow cylinder (*see* Appendix) depends neither upon the direction of diffusion (centripetal versus centrifugal), nor upon the presence of distributed sources, and cannot account for the anomalous behavior seen in the barnacle muscle fibers.

In order to see if sequestration of ^{45}Ca in the sarcoplasm could account for these observations, in one influx experiment (Fig. 6) the fiber was treated

with cyanide, oligomycin and caffeine, in an effort to dislodge ^{45}Ca which might have been taken up by mitochondria and sarcoplasmic reticulum. The fact that the ^{45}Ca influx was unaffected indicates either that these organelles did not accumulate much Ca from the 1:2 Ca/EGTA buffer, or that the treatment was insufficient to dislodge the Ca.

Effects of External Sodium and Calcium on ^{45}Ca Efflux

The original impetus for the present project was the observation that in intact, ^{45}Ca-injected barnacle muscle fibers, about half of the isotope efflux is dependent upon external Ca; a variable fraction of the Ca_0-independent Ca efflux is external Na-dependent (Russell & Blaustein, 1974). It was hoped that with internal solute control, the properties of these external cation-dependent Ca effluxes could be more completely explored; but, as the foregoing data indicate, the picture may be clouded by Ca binding in the sarcoplasm, even in internally dialyzed fibers. Despite these complications, the effects of external Ca and Na on ^{45}Ca efflux have been examined in a number of dialyzed fibers. Replacement of external Ca by Mg caused a 20% decrease in Ca efflux, on the average ($n=9$; range = 10–50% decrease). When external Na was completely replaced by Li or choline, in the absence of external Ca, the ^{45}Ca efflux fell by an average of 17% ($n=7$). In one of the seven fibers tested, removal of Na had no effect, while in one other fiber, replacement of external Na by Li caused a reversible and reproducible (in two separate one-hour trials) decline of 60% in the ^{45}Ca efflux. The magnitudes of the Na- and Ca-dependent Ca effluxes may be underestimated because the CaEGTA leak was not taken into account.

These experiments are, in general, consistent with the idea that an Na–Ca exchange mechanism may participate in Ca transport in barnacle muscle (*also see* Blaustein, Shield & Santiago, 1971; DiPolo, 1973; Blaustein, 1974; Russell & Blaustein, 1974). However, as noted below, it may be difficult to complete a definitive examination of "active" Ca transport in barnacle muscle until we obtain much more detailed information about barnacle muscle morphology and assess its implications for diffusion and transport.

Discussion

Barnacle Muscle Morphology and the Measurement of Ca Fluxes in Dialyzed Fibers

Described in the preceding sections are several unexpected and puzzling observations which were made in the course of these dialysis experiments:

1. The ^{45}Ca influx rises to a steady level much more rapidly than does the ^{45}Ca efflux in fibers of comparable diameter (*see* Tables 1 and 3, and Figs. 2*A*, 3 and 6).

2. The steady ^{45}Ca efflux is considerably larger than the steady ^{45}Ca influx measured under identical conditions (*see* Tables 1 and 3).

3. The Ca efflux is in part dependent upon $[CaEGTA]_i$ since ^{45}Ca efflux increases when the total Ca concentration is increased while $[Ca^{2+}]_i$ is maintained constant in the dialysate by buffering with EGTA (*see* Table 1 and Fig. 3).

These findings clearly require an explanation. The fact that very similar ^{45}Ca influx (DiPolo, 1973) and ^{45}Ca and ^{14}C-EDTA efflux (Vogel & Brinley, 1973; Brinley & Spangler, 1975) observations have been made in two other laboratories probably indicates that trivial errors in methodology are not responsible. Furthermore, in dialyzed squid axons ^{45}Ca influx and efflux have comparable time courses (Blaustein & Russell, 1975), and the Ca efflux in squid is not influenced by changes in the total Ca concentration of the dialysis fluid (F.J.Brinley, Jr. and L.J.Mullins, *personal communication*). Thus, artifacts in the dialysis technique *per se* do appear to be the cause of these anomalous effects.

Ca Flux Rise-time

The surface morphology differences between squid axon and barnacle muscle suggests a possible explanation for the differences in the ^{45}Ca flux behavior of the two preparations. Although the squid axon surface membrane can be reasonably well represented as a hollow right circular cylinder, the barnacle muscle sarcolemma is deeply invaginated, with major and minor clefts and narrow tubules forming a richly ramifying network throughout the muscle fiber (Hoyle *et al.*, 1973). The latter arrangement suggests that, during influx experiments, the "extracellular" space (within these clefts and tubules) might serve as a "distributed source" and cause a more rapid rise-time for influx than for efflux. However, calculations based on these diffusional considerations make it clear that influx and efflux should have the same rise-time so that the time-course difference cannot be ascribed to diffusional problems alone. We cannot yet explain why the rise-time for Ca influx (Table 3, column 8) is only slightly greater than the time constant for the washout of the extracellular spaces, 7–9 min (Russell & Blaustein, 1974).

The Magnitudes of the Steady Ca Influx and Efflux

In contrast to the situation in resting, intact muscle fibers, where the steady Ca influx and Ca efflux must be equal, we have observed a large discrepancy between the magnitudes of the unidirectional Ca fluxes in dialyzed fibers. Several underlying factors may contribute to this discrepancy. One important point is that the detailed composition of the sarcoplasmic fluid is unknown. The conditions imposed by the composition of the dialysis fluid (even with respect to $[Ca^{2+}]_i$, $[Na^+]_i$ and $[ATP]_i$) may differ significantly from those which prevail in the intact muscle fibers (the presence of EGTA and sucrose is certainly unphysiological), and these factors may influence the unidirectional ion fluxes. For example, lowering $[Na]_i$ would be expected to reduce Ca influx (Blaustein *et al.*, 1971; DiPolo, 1973) and increase Ca efflux (Vogel & Brinley, 1973). Steady unequal influxes and effluxes of ions may be maintained in the dialyzed fibers because the dialysis fluid and extracellular medium serve as infinite sources and sinks (of Ca and Na, for example); thus, the system is not "closed", in contrast to the situation in intact fibers.

Are There Standing Gradients in the Clefts and Tubules?

Another consideration concerns the morphology of the muscle fibers, particularly the narrow "minor" clefts and small tubules which may extend for several hundreds of microns into the sarcoplasm (Hoyle *et al.*, 1973). While no quantitative morphological data are available, it is apparent that the "extracellular" spaces within these clefts and tubules must have very large surface-to-volume ratios. If a significant fraction of the transsarcolemmal transport actually occurs across the surface membranes of the minor clefts and tubules, we must consider the possibility that the presence of EGTA (or EDTA) in the dialysis fluid and sarcoplasm may lead to the formation of EGTA (or EDTA) standing gradients in these interstices.

The presence of small tubules with standing gradients of EGTA may affect ^{45}Ca influx and efflux determinations in different ways. During efflux experiments virtually all of the ^{45}Ca exiting from the sarcoplasm would be expected to reach the bulk extracellular fluid surrounding the muscle fibers. Negligible back-flux of ^{45}Ca should occur because of dilution with extracellular ^{40}Ca, and because of the low $[Ca^{2+}]_0$ (due to the relatively high $[EGTA]_0$) in the confined "extracellular" spaces.

On the other hand, during influx measurements, relatively little ^{45}Ca should enter the sarcoplasm across the plasma membrane surrounding the confined "extracellular" spaces because of the low $[Ca^{2+}]_0$, and per-

haps because of reduction of extracellular ^{45}Ca specific activity due to efflux of ^{40}Ca from the sarcoplasm. In effect, more of the plasma membrane surface area may be availale for ^{45}Ca efflux than for influx. This hypothesis could, therefore, help to account for the discrepancy between ^{45}Ca influx and efflux magnitudes noted above (and *see* Tables 1 and 3).

If the $[Ca^{2+}]_0$ in the cleft and tubule spaces is reduced in dialyzed fibers, the limited sensitivity of the Ca efflux to external Ca (only about 20 % of the Ca efflux is Ca_0-dependent; p. 172) could also be explained. By comparison, in injected fibers, where negligible amounts of EGTA are introduced into the sarcoplasm, about 50 % of the Ca efflux is Ca_0-dependent (Russell & Blaustein, 1974).

Finally, a standing gradient of chelator could also contribute to the correlation between Ca efflux and total Ca concentration (*see* Table 1 and Fig. 3). Although the measured efflux of ^{14}C-EDTA was only about 10 % of the ^{45}Ca efflux (*see* p. 166 and Fig. 4), CaEDTA efflux may have accounted for more than 10 % of the ^{45}Ca efflux if a significant fraction of the EDTA was recycled (i.e., if there was much back-flux of EDTA from extracellular fluid to sarcoplasm). Back-flux would be especially likely to occur if the chelator is concentrated in the interstices. Under these circumstances, use of ^{14}C-EDTA to determine the efflux of EDTA would underestimate the actual efflux by an amount equal to the back-flux of ^{14}C-EDTA.

Unfortunately, experimental verification of the foregoing ideas would only serve to support the tentative conclusion based on the observations reported here: namely, that the absolute values of Ca fluxes obtained from internally dialyzed *Balanus* muscle fibers must be viewed with exceptional caution. Consequently, this preparation may be less than ideal for the detailed examination of Ca transport kinetics and stoichiometry.

Appendix

Diffusion in Dialyzed Barnacle Muscle Fibers

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Brinley and Mullins (1967) have discussed the representation of a dialyzed (squid) axon as a pair of concentric hollow cylinders, the inner cylinder being the dialysis tube, and the outer cylinder, the axoplasm, and they have presented numerical solutions of the resulting equations. Clearly, this model can not accurately represent the dialyzed barnacle muscle fiber because of the latter's complex invaginated surface morphology. Moreover, its solution requires computer simulation and a knowledge

of the diffusion coefficients for both the dialysis tube wall and the cytoplasm. Since the barnacle muscle presumably cannot be represented by so elementary a model, expenditure of effort for a computer simulation seemed unwarranted and we therefore adopted a grossly over-simplified model which could be treated analytically to obtain crude estimates of the diffusional characteristics of substances in the sarcoplasm.

In this model, the muscle fiber, with internal dialysis tube in place, will be represented by a single infinitely long hollow cylinder of internal radius a (the radius of the hollow core of the dialysis tube), and external radius b (the mean radius of the muscle fiber). The boundary condition at $r=a$ is taken to be $c=C_0$ where $c(r;t)$ is the concentration of label within the sarcoplasm. The boundary condition at $r=b$ is taken to be $-D'\frac{\partial c}{\partial r}=\xi$, where D' is the effective or apparent diffusion coefficient of the tracer-labeled substance into which are lumped not only the effects of normal diffusion in *both* cellulose and sarcoplasm, but also the effects (if any) of sarcoplasmic binding. The initial condition is taken to be $c(r;0)=0$. The loss of radioactive tracer in the efflux case is assumed to be negligible in comparison to the overall concentration of tracer (*cf.* Brinley & Mullins, 1967). The effect on influx of possible loading from the invaginations is assumed to be representable by a distributed source of the form $S_0\sigma(r)$ where S_0 is a constant which specifies the strength of the source and $\sigma(r)$ is a time-independent function which describes its radial variation. The set of equations to be solved is then

$$D'\nabla^2 c-\frac{\partial c}{\partial t}=-S_0\sigma \tag{1a}$$

$$c(a;t)=C_0 \tag{1b}$$

$$\left.\frac{\partial c}{\partial r}\right|_{r=b}=-\frac{\xi}{D'} \tag{1c}$$

$$c(r;0)=0. \tag{1d}$$

Eqs. (1a–d) are readily solved by the Laplace transform technique. If the Laplace transform of $c(r;t)$ is $\tilde{c}(r;p)$ and if $q^2=p/D'$, the general solution of Eq. (1a) is

$$\tilde{c}(r;p)=A(p)\,K_0(qr)+B(p)\,I_0(qr)-\frac{S_0}{D'p}\,\tilde{C}(r;p) \tag{2}$$

where

$$\tilde{C}(r;p)=-K_0(qr)\int I_0(qr)\,\sigma(r)\,r\,dr+I_0(qr)\int K_0(qr)\,\sigma(r)\,r\,dr. \tag{3}$$

By applying the boundary conditions (1 b) and (1 c) to this solution, expressions for $A(p)$ and $B(p)$ can readily be found. Thus, the final solution for $\tilde{c}(r;p)$ will be similar to that obtained by Carslaw and Jaeger (1959) for the case $S_0=0$, except:

a) There will be an additional term $-\dfrac{S_0}{D'p}\tilde{C}(r;p)$

b) In the expressions for $A(p)$ and $B(p)$, $-\dfrac{k_3}{k_2}$ must be replaced by

$$\frac{C_0}{p}+\frac{S_0}{D'p}\tilde{C}(a;p) \quad \text{and} \quad \frac{k_3'}{k_1'} \quad \text{by} \quad -\frac{\xi}{D'p}+\frac{S_0}{D'p}\left.\frac{d\tilde{C}}{dr}\right|_{r=b}.$$

Since $\tilde{C}$ and $d\tilde{C}/dr$ are single-valued and regular except perhaps at $p=0$, the inversion to obtain $c(r;t)$ will yield an ordinary residue series of the form:

$$c(r;t)=f_0(r)+\sum_{n=1}^{\infty} f_n(r)\, e^{-D'\alpha_n^2 t} \tag{4}$$

where α_n are roots of the equation

$$J_0(\alpha a)\, Y_1(\alpha b)-Y_0(\alpha a)\, J_1(\alpha b)=0 \tag{5}$$

and are entirely independent of C_0, ξ, and $S_0\sigma(r)$. That is, these quantities affect $c(r;t)$ only through the $f_n(r)$ and not through the α_n.

Since the measured flux $F(t)$ will presumably be some integral of $c(r;t)$ and $\partial c/\partial r$ over the surfaces at $r=a$ and $r=b$ and over the volume $a<r<b$, this flux should be representable as

$$F(t)/F(\infty)=1-\sum_{n=1}^{\infty} A_n e^{-D'\alpha_n^2 t}. \tag{6}$$

The α_n increase quickly with n (*cf.* Table 4) while the A_n normally decrease. Hence, for the purpose of obtaining a rough estimate of the diffusion coefficient, it often suffices to truncate the summation after one term, to define the rise-time τ as that time for which $F(t)/F(\infty)=1-e^{-1}=0.632\ldots$, to make the substitution $z_1=a\alpha_1$, and to write

$$D'=\frac{a^2}{\tau z_1^2}. \tag{7}$$

Values of $z_n(=a\alpha_n)$ satisfying Eq. (5) have been tabulated by Bogert (1951) as a function of $\lambda(=b/a)$ for $n=1$ and various values of λ, including $\lambda=2(1)10$ and $\lambda=20$. Additional values were computed here and the extended

Table 4. Roots of the equation: $J_0(z_n)\,Y_1(\lambda z_n) - Y_0(z_n)\,J_1(\lambda z_n) = 0$

λ	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75
$\lambda^{4/3} z_1$	3.429	3.132	2.939	2.804	2.707	2.634	2.577	2.533
$\lambda^{4/3} z_2$	11.707	10.933	10.463	10.163	9.969	9.843	9.763	9.716
λ	4.00	4.50	5.00	5.50	6.00	7.00	8.00	9.00
$\lambda^{4/3} z_1$	2.498	2.448	2.414	2.392	2.378	2.364	2.362	2.367
$\lambda^{4/3} z_2$	9.693	9.696	9.740	9.809	9.894	10.089	10.299	10.514
λ	10.00	12.00	14.00	16.00	18.00	20.00		
$\lambda^{4/3} z_1$	2.376	2.401	2.431	2.462	2.494	2.525		
$\lambda^{4/3} z_2$	10.727	11.140	11.532	11.902	12.251	12.581		

results are presented in Table 4. Linear interpolation within Table 4 will yield z_1 and z_2 to better than 1 % over the entire range $2 < \lambda < 20$ and z_1 to better than 0.1 % over the most important physiological range $6 < \lambda < 12$.

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