Calcium and EDTA Fluxes in Dialyzed Squid Axons

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ABSTRACT   Ca efflux in dialyzed squid axons was measured with ^{45}Ca as a function of internal ionized Ca in the range 0.005–10 μM. Internal Ca stores were depleted by treatment with CN and dialysis with media free of high energy compounds. The $[Ca]_i$ was stabilized with millimolar concentrations of EDTA, EGTA, or DTPA. Nonspecific leak of chelated Ca was measured with $[^{14}C]$-EDTA and found to be 0.02 pmol/cm^{2}s/mM EDTA. Correction of the measured Ca efflux for this leak of chelated calcium was made when appropriate. Ca efflux was roughly linear with internal free Ca in the range 0.005–0.1 μM. Above 0.1 μM, efflux was less than proportional to concentration but did not saturate at the highest concentration studied. Ca efflux was reduced about 50% by replacement of external Na with Li at $Ca_i \approx 1$ μM, but was insensitive to such replacement for Ca < 0.1 μM. Ca efflux was insensitive to internal Mg in the range 0–4 mM, indicating that the Ca pump favors Ca over Mg by a factor of about 10^6. Ca efflux was reduced about 60% by increasing internal Na from 1 to 80 mM. This effect could represent weak interference of a Ca carrier by Na or a loss of driving force because of a reduction in $E_{Na} - E_m$ occasioned by an increase in Na_i. A few measurements were made of Ca influx in intact and in dialyzed fibers. In both cases, Ca influx increased when external Na was replaced by Li.

INTRODUCTION

Previous studies of Ca fluxes in squid axons have established that in injected fibers the Ca efflux is reduced about 50% when Na and Ca are removed from the external bathing medium. However, if the axon is first treated with CN to raise the internal ionized Ca, not only does the efflux rise, but the fraction of total efflux that is sensitive to external Na + Ca increases dramatically. The Ca efflux and its Na sensitivity do not seem to require the presence of ATP since both can be observed in dialyzed axons in which the ATP has been reduced to very low levels (DiPolo, 1973); however, there is evidence that the Ca efflux is about doubled by physiological concentrations of internal ATP (DiPolo, 1974).

The present experiments were undertaken to provide more information about the concentration dependence of the Ca efflux and its Na + Ca sensi-

tivity. The technique of internal dialysis was used to introduce known concentrations of Ca and various chelators into the axoplasm. Since millimolar concentrations of chelator are needed to buffer the ionized Ca in the axoplasm adequately, the concentration of labeled Ca bound to the chelator is much greater than the free concentration; thus one cannot assume that the efflux of radioactive Ca in the chelated form is negligible compared to the total. In fact, an earlier series of experiments (Mullins and Brinley, 1975) suggested that at low concentrations of free Ca a substantial fraction of the tracer efflux was leak of chelated calcium.

In the present work, the leak of chelated calcium was estimated directly with [^{14}C]labeled EDTA and ^{45}Ca in the same axon. With correction for this leak it was possible to measure Ca efflux in the range of 0.005–10 μM. The results indicated that the Ca efflux was most sensitive to internal free Ca in the low (presumably physiological) range of 0.1–0.5 μM. Above this range the efflux was less sensitive to Ca$_i$ but did not saturate.

Several other aspects of the ionic dependence of Ca efflux were also investigated. Recent work (Mullins and Brinley, 1975) indicates that the Ca efflux is sensitive to membrane potential, as would be expected if the energy for the Ca/Na exchange were stored in the electrochemical gradient for Na, and if the exchange were electrogenic. In the previous experiments the electrical gradient was altered, whereas the present work involved changing the chemical gradient for Na (by changing Na$_i$) and observing the effect upon efflux.

Several recent reports have mentioned some similarities between Mg and Ca transport in nerve and muscle, i.e., sensitivity to external Na and internal ATP. These data are compatible with the existence of a single divalent cation pump, but there has been no report of attempts to demonstrate a direct effect of Mg on Ca efflux. Accordingly, experiments were done in which the Ca efflux was measured in the presence and in the absence of internal Mg.

Finally, in dialyzed axons, an effort was made to measure a component of the Na-dependent Ca extrusion system which, in injected axons, appears to run in the reverse of the normal direction, i.e. by extruding internal Na for external Ca (Baker et al., 1969). In ATP-free axons ($\sim$1 μM ATP) the Na efflux was reduced to nearly the level predicted by the flux ratio equation, and the excess ($\sim$0.5 to 1 pmol/cm^{2}s) was not sensitive to external Ca.

METHODS

Experimental Material

The experiments reported here were performed upon the hindmost giant axon isolated from living specimens of *Loligo pealei* during May and June 1974 at the Marine Biological Laboratory in Woods Hole, Massachusetts. Methods for isolating and cleaning the axons were standard.

Efflux Measurements

The basic technique for internal dialysis has been described previously (Brinley and Mullins, 1967). In the present work, cellulose acetate tubing (obtained from Fabric Research, Inc., Dedham, Mass.) was substituted for the porous glass previously used, and selected portions of this tubing were made permeable by 8–24 h of hydrolysis in 50 mM NaOH at 20–25°C. The permeability properties of this material have been extensively studied (Forbush, 1975) and a preliminary description published (Brinley and Mullins, 1974). The plastic tubing is approximately 25% as porous to Ca as the glass capillaries; however this disadvantage is more than offset by the lesser expense and fragility of the plastic tubing.

Since the flexibility of the plastic tubing precluded pushing it directly through the axoplasm as had been done with the glass tubing, a 100-μm tungsten wire was glued to the end of the plastic tubing with Eastman 910 adhesive (Eastman Kodak Co., Rochester, N.Y.). This wire was then guided through the axon under microscopic control. As the leading edge of the tungsten wire emerged from the end cannula, it was attached to a micromanipulator and then the plastic tubing was pulled into position.

Because the porous region increased in length approximately 10% when wet (i.e. as it was passed into the axoplasm), the capillary showed a tendency to kink inside the axon. For this reason it was found advisable to maintain the plastic tubing under as much tension as possible during the time that it was being pulled through the axon. The plastic tubing used in the efflux experiments had dimensions of 141 $\times$ 91 μm or 110 $\times$ 75 μm (OD $\times$ ID). The standard flow rate used in all experiments was approximately 1 μl/min.

Electrical Measurements

The dialysis chamber had provision for stimulation and external recording of action potentials. In addition, absolute measurements of resting or action potentials were made by means of a 100-μm glass capillary inserted longitudinally into the axon alongside the dialysis capillary. The capillary was filled with either 0.5 M or 3 M KCl, had a 25-μm platinum iridium wire in it to reduce capacitance, and was connected via a calomel electrode to the input of a high impedance, unity gain amplifier (Instrumentation Laboratory, Inc., Lexington, Mass., IL-181). The amplifier output was connected to a digital voltmeter, a chart recorder and/or a cathode ray oscilloscope. Although resting potentials were occasionally measured by coupling a 3 M KCl salt bridge to the end of the porous plastic tubing, this procedure was unsatisfactory for three reasons. First, such potentials are measured at the end of the porous region where dialysis control of internal solute composition is poor; hence the measurement does not reflect the membrane potential in the center of the dialyzed region. In addition, repeated measurements have indicated that there is a junction potential across the wall of the porous capillary of polarity such as to make the potentials recorded from inside the dialysis capillary approximately 5–10 mV more negative than those recorded from the microelectrode in the axoplasm. Finally, the "wall potential" is somewhat flow sensitive, presumably because the pressure necessary to move dialysis fluid through the capillary results in electroendosmosis.

Solutions

The external and internal solutions used in these experiments are listed in Table I. External solutions always contained 1 mM CN unless otherwise indicated. Internal solutions were deliberately made hypotonic as a result of observations in dialyzed *Myxicola* axons (Forbush, 1975) and later in dialyzed barnacle muscle fibers (Brinley and Spangler, 1975), which indicated that dialysis with isotonic solutions caused a

TABLE I

COMPOSITION OF SOLUTIONS USED

| | Concentration | | | | | |
| | External | | | | Internal | |
Constituent	Seawater (ASW)	0 Na	0 Ca	0 Na, 0 Ca	CR	CS
	mM	*mM*	*mM*	*mM*	*mM*	*mM*
K	9	9	9	9	300	380
Na	438	3	438	3	80	1
Li	0	438	0	435	0	0
Mg	21	21	30	30	0	0
Ca	8	8	0	0	0	0
Cl	505	505	507	507	0	0
Isethionate	0	0	0	0	150	190
L-Aspartate	0	0	0	0	230	190
CN	1	1	1	1	0	0
EDTA	0.1	0.1	0.1	0.1	0	0
TES*	2	2	2	2	0	0
BES*	0	0	0	0	10	10
Glycine	0	0	0	0	275	275
pH	7.5	7.5	7.5	7.5	7.0	7.0

* TES: N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

‡ BES: N,N-Bis(2-hydroxyethyl)-2-aminoethane sulfonic acid.

slow deterioration of electrical and/or transport properties which did not occur when the dialysis solutions were hypotonic. Osmolarity of the solutions was determined by comparison of the dewpoint lowering of standard and unknown solutions using a commercial psychrometer (Wescor Inc., Logan, Utah). Replicate measurements could be made at any ambient temperature on 10 λ samples with 2 % reproducibility. All seawater solutions were adjusted to 900 mosmol/kg as measured against NaCl standards and all internal dialysis solutions were adjusted to 810 mosmol/kg.

The ionized [Ca] was adjusted as follows. A stock solution of Ca chelator was prepared to contain equal amounts of free and bound chelator. The pH of this concentrated stock solution was then adjusted to pH 7.0 by titration with KOH. The nominal ionized [Ca] of this mixture would then be given by K_m for the particular chelator. Appropriate amounts of this concentrated Ca chelator solution were then added to

aliquots of dialysis solution to give the desired Ca chelator concentration in the dialysis medium. Because the stock dialysis solution contained significant amounts of Ca (approximately 40 μM) as a contaminant, particularly from isethionate, it was necessary to take account of this contaminating Ca in calculating the final level of ionized Ca. The concentration of total Ca in the standard solutions was checked occasionally by atomic absorption spectrometry.

When it was desired to change the ionized [Ca] during the course of an experiment, this was accomplished simply by adding additional aliquots of standard Ca or chelator solutions to the prepared internal dialysis solution.

Three chelators were used in this series of experiments: EGTA, EDTA, and DTPA (diethylenetriamine pentaacetic acid). Apparent dissociation constants were calculated from data given by Bjerrum et al. (1957), using the methods described by Caldwell (1970). The apparent calcium-chelator dissociation constants at pH 7.0 used were (M): EGTA, 0.21 $\times$ 10^{-6}; EDTA, 0.055 $\times$ 10^{-6}; DTPA, 3.0 $\times$ 10^{-6}. Inasmuch as these stability constants were determined in 0.1 M KCl solution at 25°C (conditions of ionic strength, composition, and temperature which differ significantly from those obtained during actual dialysis), the calculated values for ionized Ca should be considered nominal. The symbol [Ca]$_i$ will be used herein to denote the internal ionized (or free) calcium concentration. The efflux of Ca bound to a chelator will be referred to as chelated calcium leak.

The internal solutions were Mg free unless specifically indicated otherwise. Since squid axoplasm contains about 3 mM ionized Mg (Baker and Crawford, 1972; Brinley and Scarpa, 1975) which can bind to the chelators in the dialysate, the ionized [Ca] in axoplasm will be somewhat indeterminate early in the dialysis until the Mg is washed out. We have no independent measure of the washout time for Mg; however, the data of Baker and Crawford (1972) indicate that Mg is not bound in axoplasm, and the apparent diffusion coefficient is about half that found in bulk solutions. On this basis the half time for Mg washout would be about 10 min. Since all measurements of steady-state efflux were made after at least 30 min of initial dialysis, the axoplasm should have been essentially free of Mg by this time.

Tracer Methodology

Radioactive ^{45}Ca (specific activity approximately 1,000 Ci/mol) was obtained from the General Electric Company, Pleasanton, Calif. Aliquots of the stock solution were taken to dryness and added directly to aliquots of dialysis solution as necessary. The contribution of the radioactive Ca to the total [Ca] in these solutions was usually negligible, but where necessary a correction was made.

EDTA, uniformly labeled with ^{14}C (specific activity 19.8 Ci/mol) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. This was dissolved in appropriate amounts of dialysate to make the final concentration of EDTA approximately 5 mM. The EDTA was then titrated with a stoichiometric amount of standard Ca solution. This was done so that virtually all of the EDTA was present in the Ca salt, while giving an ionized [Ca] that was in the micromolar range. The pH was always adjusted to 7.0. Uniformly labeled [^{14}C]sucrose was obtained from New England Nuclear, Boston, Mass. (specific activity 4.9 Ci/mol) as the solid and dissolved in dialysate to give a final concentration of 10.3 mM.

The efflux samples, generally 2–4 ml, were combined with 10–24 ml of a commercial scintillation fluid miscible with water. The samples were counted in a liquid scintillation counter using the counter parameters for ^{14}C. Counting efficiency using this arrangement was about 40% for Ca and 80% for ^{14}C.

Influx Measurements

Although the dialysis capillary has been used for sequential measurements of influx on axons, there are limitations to the technique which have been discussed at length in the Methods sections of preceding papers (Brinley and Mullins, 1967; Mullins and Brinley, 1969). The basic problem is that it is impossible to avoid collection (by the porous capillary) of isotope which enters the nondialyzed end regions of the fiber and diffuses longitudinally into the dialyzed region in the center of the fiber.

Modification of the dialysis technique to permit influx measurements was undertaken when it was discovered that the amount of ^{45}Ca collected by the porous capillary after a period of exposure to radioactive seawater was little reduced by subsequent long washes in tracer-free seawater. This was probably due to diffusion of isotope from the fiber ends since samples of axoplasm extruded from the undialyzed end regions proved to have amounts of radioactivity which were equivalent to an apparent influx several-fold higher than that calculated for the central region. Increasing the length of the porous region to permit dialysis of the entire (or nearly entire) length of axon was of no help because then the influx included contributions from depolarized or damaged regions near the cannulated end of the fiber. Another procedure employed was to use a standard length of porous region inside the axon but shorten the length of axon exposed to seawater containing ^{45}Ca by the use of Vaseline seals in the center compartment. Thus, the length of axon exposed to seawater was only 5 mm instead of
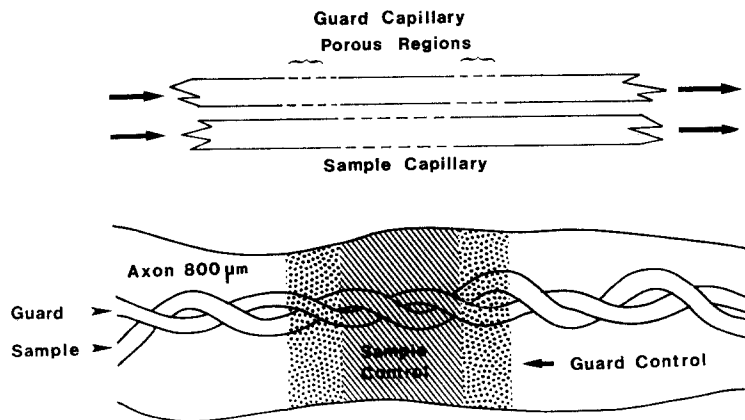

FIGURE 1.   Schematic diagrams illustrating the principle of influx measurement are shown below. The porous region of the sample capillary is 10–12 mm long and the guard region 5–7 mm. The separation between guard and sample region was 0–1 mm. The lower diagram illustrates the actual assembly with the two capillaries wound around one another with a pitch of about one turn per 5–10 mm. Inside an axon, this assembly controls the central region (hatched area) by introducing regions of solute control (dotted areas) on each side of the sample region.

15. Tests with this arrangement showed that apparent Ca influx was not reduced, and further that the application of isotope-free seawater outside had no effect on the apparent Ca influx.

The apparatus adopted to meet this problem is shown in Fig. 1. Two porous capillaries (110 × 75 μm) were incorporated into the dialysis system. The location of porous regions was adjusted so that the porous length of 10–12 mm in the sample capillary was flanked on either side by a 5–7-mm porous region in the guard capillary. The capillaries were wound around one another with a pitch of one turn per 5–10 mm, and were glued together by occasional small drops of epoxy cement.

The length of fiber exposed to tracer was limited to the central 21 mm of axon by means of the guard syringes which collected fluid from either guard slot at 10 λ/min. Tests with dye in the seawater showed a sharp barrier was maintained at the level of the guard slot. The porous regions in the guard capillary extended about 1–3 mm beyond the portion of axon exposed to isotope to reduce longitudinal gradients in the axoplasm.

The results of separate influx experiments using the single and double capillaries are compared in Fig. 2. The axons had nearly identical diameter so that equilibration time would be the same. The fibers were exposed to ^{45}Ca seawater and samples of the effluent dialysate collected until isotopic equilibration had been reached (vertical arrow and "X"). The external solution was then replaced with nonradioactive seawater. The radioactivity of the effluent dialysate in the double capillary system decreased with a half time essentially the same as the half time of equilibration, while
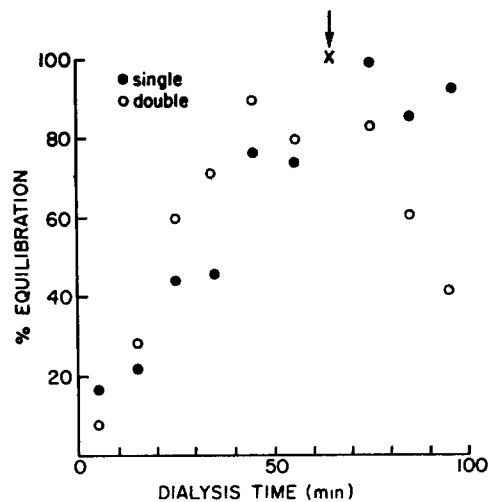

FIGURE 2. This shows the performance of a single vs. a double capillary used for Ca influx measurements with two different axons. A reference time of 65 min (shown by X) is chosen for 100% equilibration after which ^{45}Ca is removed from outside the axon. Note that equilibration proceeds at about the same rate for single and double capillaries, but that upon removal of isotope outside, only the center section of the double capillary shows a decline in counts. External solution: ASW; internal solution: CS + 1 mM EGTA.

the radioactivity in the effluent from the single capillary system was unchanged after 30 min of dialysis in nonradioactive solution. Analysis of the counts in the end regions of this axon showed the axoplasm to be highly radioactive.

RESULTS

EDTA Efflux

A previous study (Mullins and Brinley, 1975) strongly suggested that part of the measured Ca efflux was not a result of Ca^{++} being transferred by the Na/ Ca exchange system, but was a consequence of the use of a chelating agent such as EGTA. If some form of CaEGTA can cross the membrane, then the contribution of this moiety to the total Ca flux must be allowed for in the measurements made. Since [^{14}C]EGTA was not readily available, [^{14}C]EDTA was used to measure leak of chelated calcium.

Fig. 3 illustrates an experiment in which an axon was dialyzed with solution CS plus 1.25 mM each of Ca and EDTA. The calculated free [Ca] and [EDTA] for such a stoichiometric mixture is 7.9 μM. [^{14}C]EDTA efflux rose to a steady plateau, was insensitive to the removal of external Ca, but was increased by applying Na-free seawater containing Li. Once a steady state was reached, the efflux was stable for several hours. Other experiments
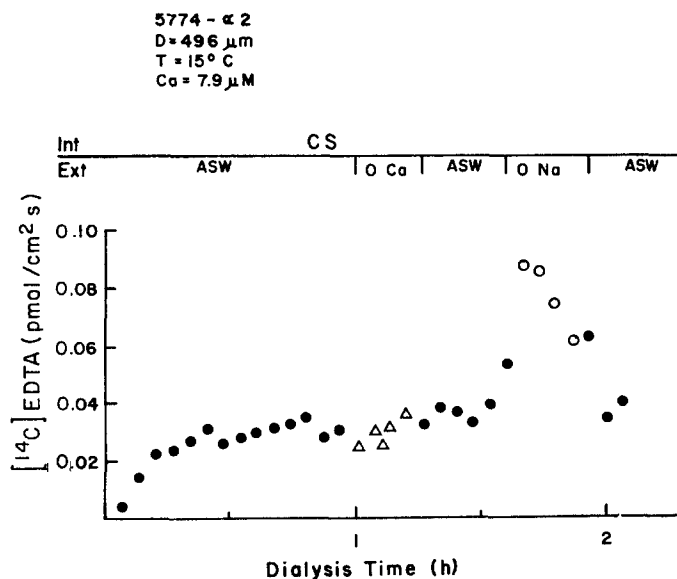

FIGURE 3.   Effect of various external solutions upon the efflux of EDTA. The efflux was unaffected by removal of external Ca, but was increased by removal of external Na. The total Ca and EDTA concentrations were each 1.25 mM, with nominal [Ca]$_i$ and [EDTA]$_i$ of 7.9 μM each. Solid circles: efflux into ASW; triangles: efflux into 0 Ca external solution; open circles: efflux into 0 Na external solution.

showed that EDTA efflux was unaffected by a membrane hyperpolarization of 30 mV.

We have no ready explanation for the increase in EDTA efflux when Na-free seawater is applied. It cannot be a result of the large increase in Ca influx produced by Na-free solutions (Baker et al., 1969), because entering Ca would find no free EDTA with which to complex since substantially all EDTA was supplied as CaEDTA.

The collected measurements of EDTA efflux on 13 different axons are shown in Fig. 4 where the flux is plotted as a function of [CaEDTA]$_i$. In all of these experiments the total Ca and total EDTA concentrations were equal.
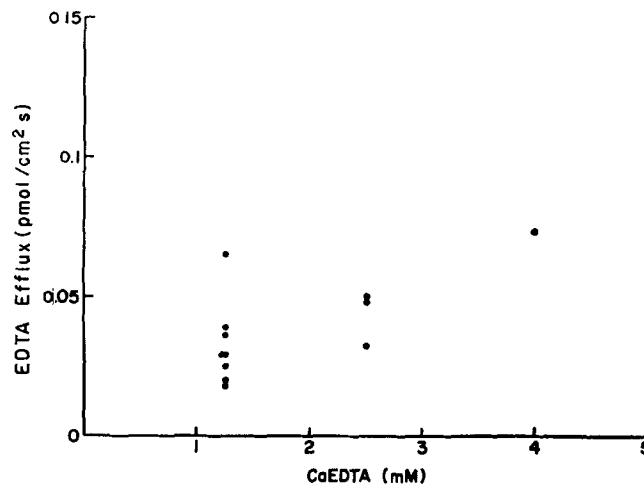

FIGURE 4.   Collected results of experiments on dialyzed axons illustrating [^{14}C]EDTA efflux as a function of various [CaEDTA]$_i$. Temperature = 17°C.

Although there is some variation of the nominal [Ca]$_i$ and free EDTA under these circumstances, the unchelated EDTA concentration was always less than 1% of the total EDTA. The mean efflux per millimolar CaEDTA was 0.024 pmol/cm^{2}s.

Sucrose Efflux

No reference has been made to the mechanism of leak of EDTA from axons. It could be related to the "leakage" conductance of squid axons and could involve the nonspecific loss of all cell constituents through cut branches and other imperfections in the membrane. A previous study (Mullins, 1966) suggested that the magnitude of membrane leakage can be estimated by measuring the efflux of sucrose, on the basis that the permeability of sucrose across the intact membrane is so low that measurable fluxes are only given if there are holes or imperfections in the membrane.

Sucrose efflux was measured on two axons using the same dialysis medium employed for Ca efflux measurements. Sucrose-^{14}C concentration was 10.3 mM for both axons. Fig. 5 shows the efflux in one experiment which, at the plateau, was 0.50 pmol/cm²s. The mean value for both axons was 0.047 pmol/cm²s per millimolar sucrose and compares reasonably well with the mean value for [^{14}C]EDTA efflux of 0.024 pmol/cm²s per mM.

Combined EDTA and Ca Efflux Measurement

Because it was suspected that the leak of chelated calcium would be a significant fraction of the total ^{45}Ca efflux at low ionized [Ca], most of these experi-
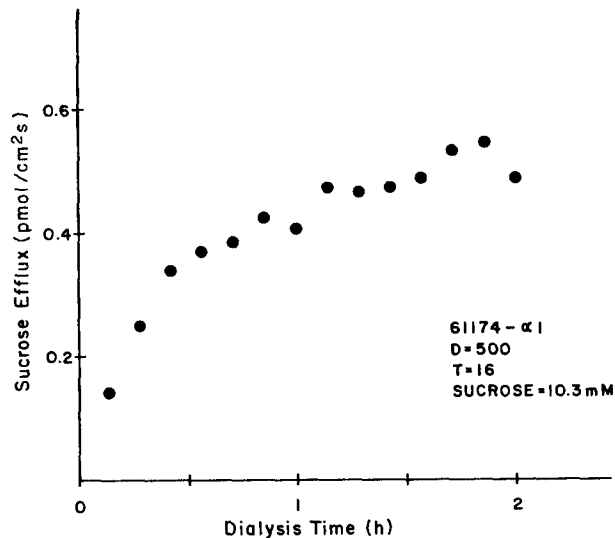

FIGURE 5. Efflux of sucrose from a squid axon dialyzed with medium "CS" and [^{14}C]-sucrose.

ments were designed such that a sequential determination was carried out, first of [^{14}C]EDTA efflux and then of ^{45}Ca efflux.

One such experiment is shown in Fig. 6. The axon was dialyzed with Ca-[^{14}C]EDTA (4 mM) for 1 h; the steady-state efflux of 0.076 pmol/cm²s was taken as the leak of chelated Ca. Subsequently, a ^{45}Ca solution buffered with EDTA/CaEDTA (EDTA$_{total}$ 1.25 mM) to a [Ca]$_i$ of 0.15 μM was introduced and ^{45}Ca efflux measured. The value of ^{45}Ca efflux measured at the plateau was 0.16 pmol/cm²s, or 0.14 corrected for chelator leak. The points plotted in Fig. 6 represent the radioactive counts of either ^{14}C or ^{45}Ca collected during each sample period. The specific activity of the [^{14}C]EDTA was adjusted relative to that of the ^{45}Ca, so that after the change to radioactive Ca, the collected radioactivity would represent predominantly Ca efflux.
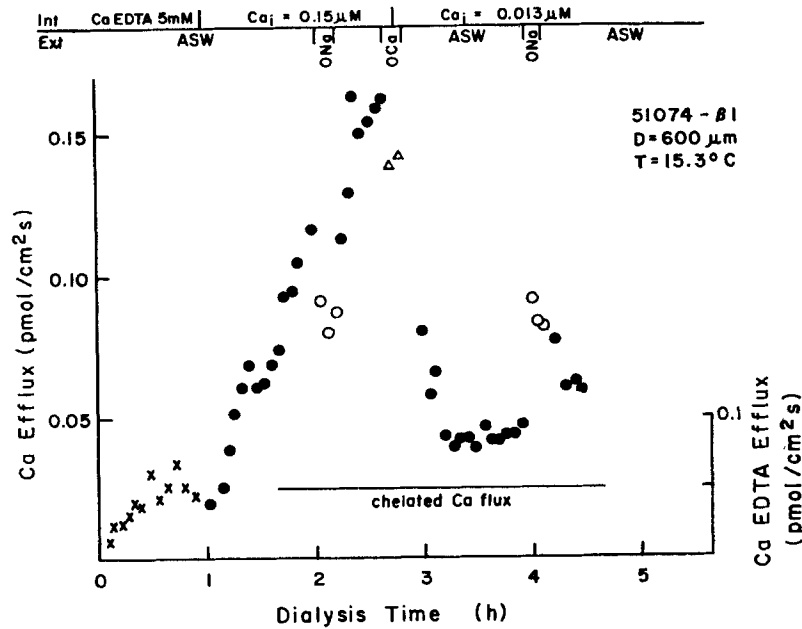
FIGURE 6.   Sequential measurement of EDTA and Ca efflux on a dialyzed squid axon. Lefthand abscissa and circles or triangles: ^{45}Ca efflux. Righthand abscissa and x: [^{14}C]EDTA efflux. Legend at top indicates sequence of internal and external solution changes. Solid circles: efflux into ASW; open circles: efflux into 0 Na external solution; triangles: efflux into 0 Ca external solution.

Since the specific activities were not comparable, different flux scales are provided for the two species. Fig. 6 also shows the effect of lowering the internal ionized [Ca] and will be discussed further in the next section.

Ca Efflux as a Function of [Ca]$_i$

At present there are only indirect estimates of the free or ionized [Ca] in axoplasm; these suggest that the concentration may be between 10^{-7} and 10^{-6} M. Because of this uncertainty, it seemed important to make measurements of Ca efflux over a wide range of free Ca in axoplasm. We have collected measurements for Ca efflux over [Ca]$_i$ of from 5×10^{-9} to 10^{-5} M. These values for Ca efflux have all been corrected for leak of chelated Ca. The results show that a net Ca efflux in excess of chelator leak is measurable over this range.

The method used for evaluating Ca efflux as a function of [Ca]$_i$ is illustrated by three experiments shown in Figs. 6, 7, and 9. Fig. 6 shows the effect of reducing ionized Ca to a very low level (nominally 0.013 μM). The flux (corrected for leak of chelated Ca) at 0.15 μM was 0.14 pmol/cm^{2}s and fell to 0.015 pmol/cm^{2}s at 0.013 μM, showing that the Ca flux was roughly proportional to ionized [Ca] in this concentration range.

Fig. 7 illustrates an experiment in which internal ionized Ca was raised from 0.1 to 1 μM. The net efflux corrected for the chelated calcium leak (as determined with [^{14}C]EDTA in the early part of the experiment) went from 0.075 to 0.27 pmol/cm^{2}s, or about a 4-fold increase in efflux for a 10-fold increase in concentration. This experiment also shows that the Na + Ca sensitivity of the Ca efflux increases with increasing [Ca]$_i$. Since the efflux at higher [Ca]$_i$ showed the greater sensitivity late in the experiment (after 6 h of continuous dialysis), it cannot be argued that the small reduction seen with 0.1 μM ionized Ca after only 3 h of dialysis represented the response of a deteriorated axon.

In another experiment (see Fig. 9), the initial [Ca]$_i$ of 0.62 μM was reduced to 0.07 μM, and the corrected flux was reduced about four- to fivefold for this ninefold reduction in free Ca. Fig. 9 will be discussed further in the section considering the effect of [Na]$_i$ on Ca efflux.

Fig. 8 presents the collected data for all experiments in which Ca efflux (corrected for leak of chelated Ca) was measured in our standard artificial seawater. These show clearly that the Ca efflux increases less than linearly above about 0.5 μM and becomes relatively insensitive to ionized Ca in the range 1–10 μM, although the efflux does not saturate because, as Blaustein and Russell (1975) have recently shown, Ca efflux rises slowly with [Ca] even up to 500 μM [Ca]$_i$.
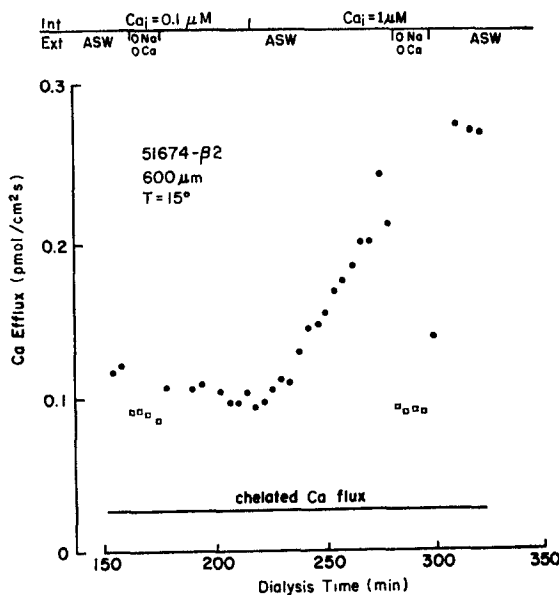

FIGURE 7. Effect of changes in internal ionized Ca on Ca efflux in a dialyzed squid axon. The horizontal bar at the bottom of the figure represents the "chelator flux" for this axon as determined during the initial 150 min of dialysis. Squares: efflux into 0 Na, 0 Ca external solution. Circles: efflux in to ASW.
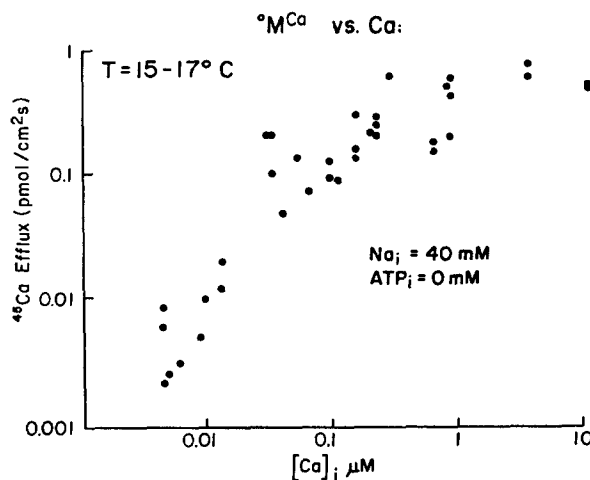
FIGURE 8.   Collected results of 36 measurements of ^{45}Ca efflux from dialyzed squid axons as a function of $[Ca]_i$. All points have been corrected for chelator leak. Values of $[Ca]_i$ are nominal (i.e. not corrected for ionic strength). The magnitudes of the effluxes at concentrations less than 0.05 μM could be in error by a factor of 2 because of large chelator leak corrections. $ATP_i = 0$; $Na_i = 40$ mM.

In collecting Ca efflux data at concentrations above 0.1 μM ionized Ca, the usual chelator concentration was 1–1.5 mM EGTA, and the chelated Ca leak was calculated on the basis of 0.024 pmol/cm²s per mM, which was the mean value for the experiments listed in Table II. At $[Ca]_i$ lower than 0.1 μM, the chelator leak could be a considerable fraction of the total efflux; therefore several-fold lower concentrations of chelator were generally used in such experiments, and in addition the leak of chelated Ca leak was measured for each axon during a predialysis with Ca-[^{14}C]EDTA. Because the approach to a steady state is sometimes very slow with low concentrations of chelator, these experiments were usually begun with a 20–30-min predialysis with 1 mM chelator in nonradioactive dialysate to remove any free Mg and to stabilize $[Ca]_i$ at the desired level. At the lowest [Ca] (below 0.05 μM), the chelator flux was about half of total Ca efflux, and the efflux of ionized Ca (measured as the difference between two numbers of about the same magnitude) could be in error by a factor of two.

It may be asked whether these values for Ca efflux at low internal ionized Ca are actually chelated Ca leak that has not been properly allowed for, rather than net Ca transport. The most convincing argument that can be given is that for low $[Ca]_i$, Ca efflux (at constant buffer concentration) varied 10-fold with a 10-fold change in ionized Ca (see Fig. 8).

As has been noted previously (Mullins and Brinley, 1975), the sensitivity of Ca efflux to Na_o and Ca_o is most impressive when $[Ca]_i$ is in the range 10–100 μM. It is substantial when $[Ca]_i$ is in the range of 0.2–1 μM, while it is usually

TABLE II

[^{14}C]EDTA EFFLUX, T = 15–17°C

Axon reference	Axon diameter	[CaEDTA]	Ca$_i$ or [EDTA]$_i$*	[^{14}C]-EDTA efflux into seawater	[^{14}C]-EDTA efflux/ [CaEDTA]$_i$	Ratio of ^{14}C effluxes		
						$\dfrac{0\text{-Ca}}{\text{Na}}$	$\dfrac{\text{Li}}{\text{Na}}$	$\dfrac{\text{Li, 0-Ca}}{\text{Na}}$
	μm	mM	μM	$pmol/cm^2 s$	$pmol/cm^2 s/mM$			
050674-1α	575	2.5	11	0.05	0.020			1.0
050674-2α	700	2.5	11	0.047	0.019			
050674-1β	700	2.5	11	0.032	0.013			1.0
050774-1α	625	1.2	8	0.029	0.023	0.73		0.7
050774-2α	496	1.2	8	0.039	0.031	0.87	1.70	
050774-1β	515	1.2	8	0.029	0.023	0.90	1.32	
050874-1α	575	1.2	8	0.020	0.016			
050974-1α	475	1.2	8	0.065	0.052			
050974-2α	600	1.2	8	0.025	0.020			
050974-1β	475	1.2	8	0.036	0.028			
050974-2β	550	1.2	8	0.018	0.015			
051074-1α	600	5.0	16	0.13	0.026			
051074-1β	600	4.0	14	0.076	0.018			

Mean = 0.024

* Nominal concentration.

absent at a [Ca]$_i$ of below 0.1 μM. A summary of our experience with the Na$_o$ and Ca$_o$ sensitivity of Ca efflux is given in Table III where axons have been grouped in order of ascending [Ca]$_i$. At a [Ca]$_i$ of 0.0004–0.01 μM, Ca efflux into Li, 0-Ca seawater is larger than into seawater, while at [Ca]$_i$ of 0.6–3.6 μM there is a substantial reduction in Ca efflux into Li, 0-Ca seawater.

The lack of saturation of Ca efflux with [Ca]$_i$ makes it impossible to give a conventional K_m that will describe the curve. What can be noted is that over a range of [Ca]$_i$ 0.01–0.5 μM, efflux is a linear function of concentration, while from [Ca]$_i$ 1–10 μM, Ca efflux only about doubles for a 10-fold increase in concentration.

If a [Ca]$_i$ of 1 μM is taken as the maximum value of physiological interest, Ca efflux is half the value for 1 μM at a [Ca]$_i$ of 0.3 μM.

Effect of [Na]$_i$ on Ca Efflux

Previous studies (Mullins and Brinley, 1975) have shown that Ca efflux is sensitive to E_m; it is increased by hyperpolarization and decreased by depolarization. This sort of response would be expected if Ca efflux depended upon the electrochemical gradient for Na. Since the electrochemical gradient for Na in a fresh axon in seawater is ~100 mV, a hyperpolarization of the membrane by 25 mV would increase the Na gradient about 25%. Experimentally, the Ca efflux was increased almost 270% by a hyperpolarization of 25 mV, suggesting that the effect of potential was not to increase the Na

TABLE III

SENSITIVITY OF Ca EFFLUX* TO Na_o AND Ca_o

$T = 16°C$, $[Na]_i = 40$ mM

Axon reference	Axon diameter	Ca buffer (X)	[CaX]	[X]	$[Ca]_i$	Ca efflux into seawater	Ratio of Ca effluxes		
							$\dfrac{0\text{-Ca}}{Na}$	$\dfrac{Li}{Na}$	$\dfrac{Li, 0\text{-Ca}}{Na}$
	μm		mM	mM	μM	$pmol/cm^2 s$			
050974-2β	550	EDTA	1.15	7.29	0.008	<0.01		1.4	
051074-1β	600	EDTA	1.75	6.82	0.013	0.012	1.00	1.47	
051074-2β	605	EDTA	0.88	6.54	0.007	<0.01			4.50
052174-1β	525	EDTA	0.08	0.92	0.005	0.0082			0.50
			0.08	10.9	0.0004	0.002			0.64
052274-1β	650	EDTA	0.08	0.92	0.005	0.012			0.54
060674-1α	600	EDTA	0.08	0.92	0.005	0.02			1.2
					(0.0004-0.01)	0.0106	1.0	1.4	1.5
050974-1α	475	EDTA	1.25	0.94	0.067	1.4		1.39	1.23
050974-1β	475	EDTA	1.25	0.94	0.067	0.11	0.87	0.68	0.53
050974-2β	550	EDTA	1.15	1.04	0.055	0.08	0.71	0.90	
050974-2α	610	EDTA	1.25	0.94	0.067	0.23	0.89	1.53	
053074-2α	575	EDTA	0.74	1.14	0.033	0.20			0.67
					(0.03-0.07)	0.404	0.8	1.1	0.8
051074-1α	600	EDTA	1.75	0.58	0.15	0.17	1.0	1.73	
051074-1β	600	EDTA	1.75	0.58	0.15	0.13	0.90	0.73	
051674-2β	600	EGTA	1.87	3.89	0.1	0.14			0.73
051774-1β	500	EGTA	1.87	3.91	0.1	0.15			
051774-2β	575	EGTA	1.87	3.91	0.1	0.20			
052474-1α	625	EGTA	1.29	1.21	0.21	0.46			
052474-1β	550	EGTA	1.29	1.21	0.21	0.35		1.0	
052474-2α	600	EGTA	1.29	1.21	0.21	0.51			0.61
052574-1α	560	EGTA	1.31	1.19	0.22	0.22		1.0	
			4.31	4.44	0.22	0.32		1.1	
052574-1β	500	EGTA	7.31	7.3	0.22	0.29		0.87	
052674-1β	575	EGTA	1.31	1.19	0.21	0.18		1.0	0.72
060574-1α	500	EGTA	0.99	0.75	0.29	0.60			0.60
					(0.1-0.3)	0.29	1.0	1.1	0.7
051674-2β	600	EGTA	1.90	0.46	0.86	0.35			0.29
051774-1β	500	EGTA	1.87	0.46	0.82	0.58			0.25
051774-2β	575	EGTA	1.87	0.46	0.82	0.71			0.28
051874-1β	700	EGTA	1.87	0.46	0.82	0.50			0.20
052674-1α	525	DTPA	1.31	1.20	3.6	0.70		0.71	0.41
052674-1β	575	DTPA	1.31	1.20	3.6	0.59		0.58	0.56
052874-1α	545	EGTA	0.95	1.25	0.62	0.15		0.87	0.53
					(0.6-3.6)	0.51		0.72	0.36

* Corrected for leak of chelated Ca.

gradient so much as to reduce some rate-limiting step in the Ca transport reaction. An example of such a step would be the facilitation of the movement of a carrier that took 3 Na inward and moved 1 Ca outward.

In the present experiments, the chemical gradient for Na was altered by changing $[Na]_i$. Fig. 9 illustrates one such experiment in which the effect of Na_i upon net Ca efflux was examined at both low (0.07 μM) and high (0.62 μM) Ca_i. The initial level of 0.25 pmol/cm²s obtained with a $[Ca]_i$ of 0.62 μM and Na_i of 80 mM increased almost threefold when Na_i was removed. A
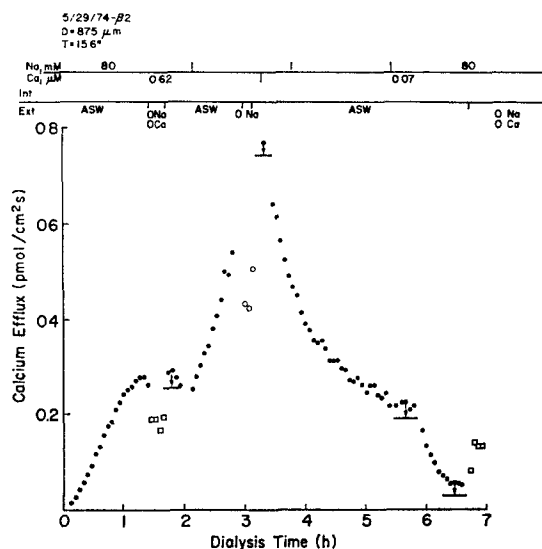
FIGURE 9. Effect of $[Na]_i$ and $[Ca]_i$ on Ca efflux. The horizontal bars and vertical arrows indicate the approximate leak of chelated Ca for each of the steady states obtained during the experiment. The single point plotted at the change from 0.62 to 0.07 mM $[Ca]_i$ represents the mean of several pooled samples. Solid circles: efflux into ASW; open circles: efflux into 0 Na external solution; squares: efflux into 0 Na, 0 Ca external solution.

similar inhibitory action of internal Na was seen later in the experiment after the Ca_i had been reduced to 0.07 μM.

Such a finding would seem to clearly rule out the idea that the measured Ca efflux at 0.07 μM was a leakage or nonspecific loss of Ca as there is no reason to believe that such a flux should be Na_i sensitive.

The results obtained from several experiments conducted at three different $[Ca]_i$ are shown in Fig. 10 where the Ca efflux at $[Na]_i$ = 1 mM is normalized to 1 and the inhibition produced by increasing $[Na]_i$ is plotted. The solid line on the curve is the change in the electrochemical gradient for Na produced by increases in $[Na]_i$. While there is substantial scatter in the data, the efflux is reasonably represented by this function; the inhibition of Ca efflux appears independent of $[Ca]_i$.

Effect of $[Mg]_i$ on Ca Efflux

In the experiments so far described, the dialysis medium contained no Mg. Under physiological conditions, however, Mg is actually present in axoplasm at a concentration of about 3 mM (Baker and Crawford, 1972; Brinley and Scarpa, 1975). Since this concentration is almost four orders of magnitude greater than that of ionized [Ca], it seemed reasonable to see if Mg in physiological concentrations might affect Ca efflux.

One of the four experiments to test the effect of various concentrations of ionized Mg is illustrated in Fig. 11. The initial dialysate contained 0.21 μM free Ca and no Mg. After a steady-state efflux was obtained, the dialysate was replaced with one of the same composition except for the addition of 5 mM total Mg. The calculated equilibrium concentrations of free ions in the second dialysate were: Ca, 0.27 μM; Mg 4.75 mM. Ca efflux increased from 0.48 to 0.61 pmol/cm²s but some increase is to be expected because of the 30%
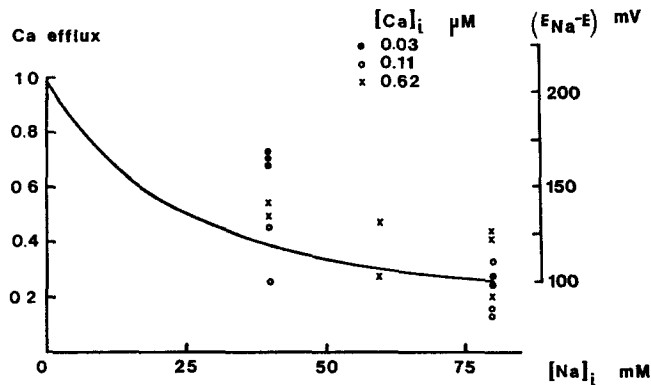

FIGURE 10. Collected data showing the net Ca efflux at various [Na]$_i$ relative to that for [Na]$_i$ = 1 mM for three values of [Ca]$_i$. The solid line represents the Na electrochemical gradient as a function of [Na]$_i$ with E_m taken as −60 mV.

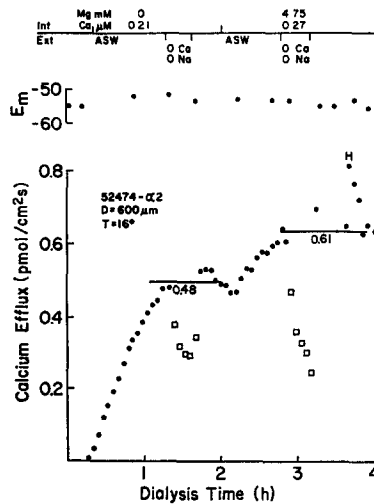FIGURE 11. Effect of [Mg]$_i$ upon Ca efflux. All effluxes have been corrected for chelator leak. Values for [Mg]$_i$ and [Ca]$_i$ are nominal. During sample marked "H," membrane hyperpolarized 28 mV (not shown on voltage record). Squares: efflux into 0 Ca, 0 Na external solution; circles: efflux into ASW. Lines represent estimates of steady-state efflux into ASW.

increase in free Ca occasioned by the addition of Mg. To allow for this change in free Ca, it seems reasonable to linearize Ca efflux vs. $[Ca]_i$ over the small range of increase. When this is done, the calculated Ca efflux in the presence of 4.75 mM free Mg is 0.52 pmol/cm²s.

The data on all four experiments are set out in Table IV. The collected results indicate about a 10% increase in Ca efflux in the presence of milli-molar concentrations of Mg, but the difference is not statistically significant.

TABLE IV

EFFECT OF $[Mg]_i$ ON Ca EFFLUX

Axon reference	$[Ca]_i$	$[Mg]_i$	Ca efflux	
			Observed	Corrected to initial $[Ca]_i$
	μM	μM	$pmol/cm^2s$	
052374-1α	0.0048	0	0.0015	
	0.0052	1,700	0.0019	0.0017
	0.022	0	0.21	
	0.024	1,900	0.26	0.24
052474-1β	0.22	0	0.35	
	0.27	4,800	0.43	0.35
052474-2α	0.21	0	0.48	
	0.27	4,750	0.61	0.52
052574-1α	0.19	0	0.52	
	0.23	3,730	0.64	0.53

Effect of La^{+++} on Ca Efflux

It has been known for some time that La^{+++} is a strong inhibitor of Ca efflux in squid axons (Van Breemen and De Weer, 1970). Fig. 12 shows this effect in a single experiment in an axon dialyzed with a $[Ca]_i$ of 0.86 μM. 1 mM La^{+++} reduced the efflux more than 90%, which was a significantly greater reduction than that produced by the removal of external Ca and Na.

Ca Influx

In the Discussion section it will be argued that an estimate of internal ionized Ca can be obtained by comparing Ca influx in intact axons under standard conditions with the Ca efflux measured at various $[Ca]_i$ in dialyzed axons. To support this argument, in addition to Ca fluxes in intact axons with a presumably "physiological" ionized Ca, it is necessary to have some evidence that Ca influx in dialyzed axons is comparable.

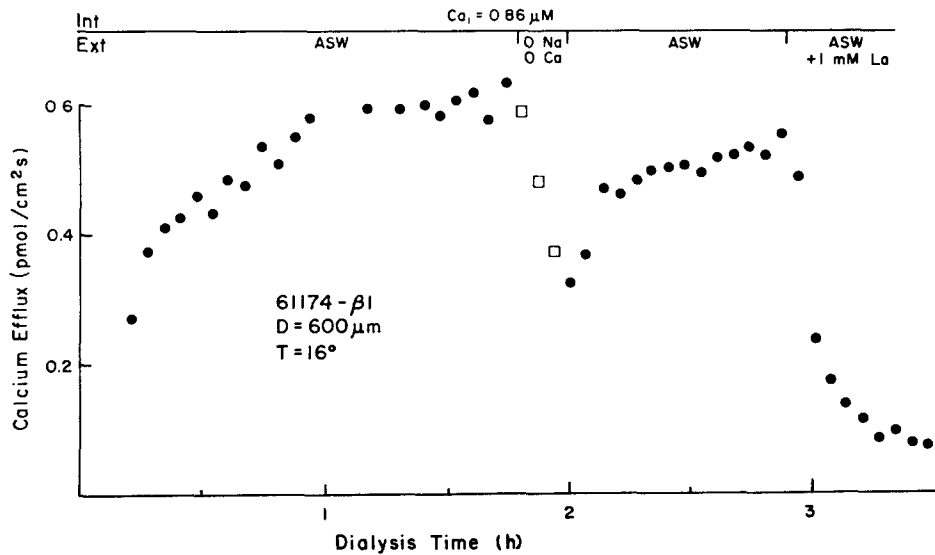Data bearing on these two points are collected in Table V. Only two meas-

FIGURE 12. Effect of 1 mM La upon Ca efflux in dialyzed squid axon. Internal solution: Na 40 mM, Ca 0.86. Squares: efflux into 0 Na, 0 Ca external solution.

TABLE V

Ca INFLUX IN SQUID AXONS (16°C)

Axon reference	Condition	Ca influx	
		Na seawater	Li seawater
		$pmol/cm^2s$	
060174-1*	Intact	0.10	
060174-2*	Intact	0.23	
060174-3‡	Intact		3.8
060174-4‡	Intact		1.5
060374-1α§	Dialyzed	0.43	
060474-1α§	Dialyzed	0.29	0.45
060474-2α§	Dialyzed		0.66

* Stored 2 h at 5°C.
‡ Stored 5–6 h at 5°C.
§ [Na]ᵢ 80 mM; CaEGTA 1.2 mM; [Ca]ᵢ 0.21 μM.

urements of influx in intact axons in seawater were made because the results, 0.10 and 0.23 pmol/cm²s, were so close to the mean of previous series in *Loligo forbesii* (Hodgkin and Keynes, 1957; Baker et al., 1969) that further confirmation of the equality of influx in the two species was not necessary.

Three measurements of influx in dialyzed axons were made and are listed in Table V. The dialysate in all three experiments contained 80 mM Naᵢ to mimic the [Na] in stored axons. The dialysate also contained 1 mM CaEGTA

to ensure that tracer Ca entering the fiber would exchange with nonradioactive Ca chelated to EGTA and be washed out of the fiber rather than be sequestered just inside the membrane. Such sequestration would make the measured influx spuriously low. The ionized Ca was set at 0.21 μM so as to be in the physiological range.

Fig. 13 illustrates one of the influx experiments. After an initial period in CN-containing, nonradioactive seawater (to reduce ATP), the axon was bathed with Li seawater for 1.5 h, during which time the influx rose to 0.45
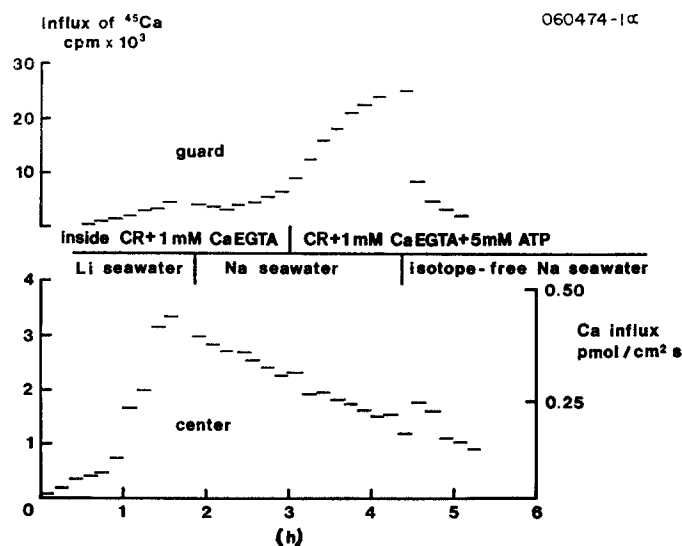

FIGURE 13.   Calcium influx in squid axons dialyzed with the double capillary system. Sampling interval is 10 min. Top: counts collected in effluent from guard capillary. Bottom: counts collected from sample capillary. Axon bathed in CN-containing seawater during entire experiment and during 1-h predialysis. Note difference in ordinate scale (cpm) for guard and center capillaries. Internal solution changes apply only to central region. Guard region continuously dialyzed with solution CS.

pmol/cm²s. The external bathing solution was then replaced by a CN, Na seawater and the influx was gradually reduced to 0.29 pmol/cm²s.

At the end of the experiment when external radioactivity was removed, the counts collected from the guard regions dropped promptly whereas those from the central region showed a gradual reduction. Presumably this difference was related to the presence of 5 mM ATP in the dialyzed region. The circumstance should have permitted mitochondrial sequestration of ^{45}Ca during the 4.25-h exposure to isotope, and its subsequent slow release. In contrast the end regions (kept in CN seawater without internal ATP) would be expected to lose isotope much more rapidly.

During the initial part of this experiment the counts collected by the guard capillary were two to three times higher than those obtained from the center

capillary, and increased steadily in the latter part of the experiment. Presumably this difference between guard and center regions represents a greater initial Ca permeability at the ends of the fiber, with a subsequent deterioration in the end regions.

Ca-Dependent Na Efflux

A system that utilizes the Na gradient to extrude Ca, in its simplest form, can be expected to run backward and extrude Na when taking up Ca. Indeed, Baker et al. (1969) have shown that in injected axons there is a Ca_o-dependent Na efflux into Li seawater when $[Na]_i$ is high. The observed Na efflux was reduced by CN and it was suggested either that ATP was required for the Na extrusion, or that the increase in $[Ca]_i$ known to occur upon application of CN inhibited the Na efflux.

Since a Na-dependent Ca efflux occurs in axons with ATP_i reduced to a level of a few micromolar, if the Ca_o-dependent Na efflux reflects the operation of the same carrier in the reverse direction, this latter flux must also be ATP independent. The present experiments were designed to investigate this point directly by testing the sensitivity of the Na efflux to Ca_o in normal and Na-free seawater in axons in which virtually all of the ATP had been removed by dialysis.

Axons were bathed in seawater containing 1 mM CN and dialyzed with cold CS + 0.1 mM EDTA for 1 h before Na efflux measurements were begun with ^{22}Na. Fig. 14 shows the results of such an experiment. Sodium

052274-2 α

Na Efflux
pmol/cm² s

Na 80mM + EDTA 0.1mM + CN 1mM   In

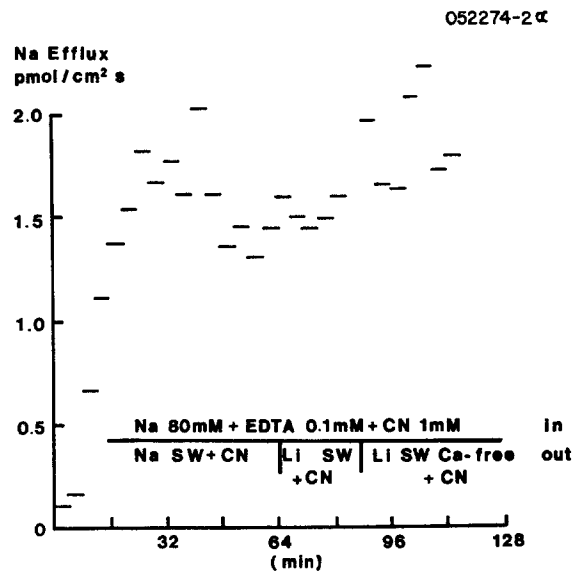Na SW+CN | LI SW +CN | LI SW Ca-free + CN   out

(min)

Figure 14. Na efflux into seawater containing 10 mM Ca or zero Ca is shown as a function of time. Axon was predialyzed in CN to reduce ATP.

efflux into seawater was about 1.5 pmol/cm²s, a value reached when [ATP]ᵢ is ∼1–2 μM.

This small Na efflux, which has been identified as passive in the Ussing flux ratio sense (Brinley and Mullins, 1968), was unaffected by removal of external Na and was possibly increased about 0.5 pmol/cm²s by removal of both Na and Ca. The collected data on three axons are given in Table VI.

TABLE VI

Na EFFLUX FROM SQUID AXONS (17°C)

Axon reference	Axon diameter	Sodium efflux			
		Na seawater	Li seawater	Li SW Ca-free	Na SW Ca-free
	μm		pmol/cm²s		
052274-1α	675	2.3	2.3	1.3	
052274-2α	925	1.5	1.5	2.0	
052274-2β	925	1.9	—	1.8	1.9

DISCUSSION

Leak of Chelated Ca

In this work, the efflux of [¹⁴C]EDTA was assumed to be a measure of the nonspecific leak of chelated Ca for EGTA and DTPA as well as for EDTA. Because it was only a small fraction of total Ca efflux, the exact magnitude of this leak was not important for internal ionized [Ca] above 0.08–0.1 μM where EGTA and DTPA were used as Ca buffers. However the correction became progressively more important as ionized Ca was lowered; at the lowest concentrations, the leak typically amounted to 50% of the total Ca efflux. (At very low concentration, internal EDTA was reduced to 250–500 μM, but this still entailed a correction of 0.005–0.01 pmol/cm²s from a total efflux of about 0.02 pmol/cm²s.) In about half of these latter experiments, EGTA was used as buffer and the leak of chelated Ca appropriate for EDTA was subtracted. Omission of these points does not significantly alter the shape of the curve.

It is possible that measurement of the efflux of [¹⁴C]labeled EDTA actually overestimates the chelated Ca leak since some of the [¹⁴C]EDTA exists uncombined with Ca. Although the concentration of the free forms is only about 1% of the chelated ones under the condition of these experiments (1 mM CaEDTA, 10 μM EDTA), the permeability, particularly of H₄EDTA, undoubtedly is much greater than any of the ionized chelated forms and might make a contribution to the [¹⁴C]EDTA efflux but not to the [⁴⁵Ca]EDTA efflux. It is impossible to estimate this sort of error without detailed knowledge of the membrane permeability to all the free and combined species.

These arguments assume that the chelator was complexed entirely with Ca and not at all with Mg, an unlikely situation early in the dialysis when the axoplasm contains free Mg. However, the leaks reported in this paper were all steady-state values obtained after long dialysis when internal free Mg should have been reduced to low levels.

Ca-Dependent Na Efflux

The absence of a Na efflux that depends on Ca_o (in the absence of ATP_i) contrasts sharply with the results of Baker et al. (1969) who found a Ca_o-dependent Na efflux of 20–30 pmol/cm²s in injected axons when $[Na]_i$ was high and Li was the principal cation in seawater. Baker et al. were able to show a large Ca influx under these conditions, and suggested that in their experiments the Na/Ca exchange mechanism was running in the reverse of the usual direction, i.e. moving Ca inward and Na outward. The present experiments show that the Na/Ca exchange mechanism does not run backward in the absence of ATP.

A summary of the effects of ATP on Ca and Na fluxes is given in Table VII. All fluxes given in this table are ouabain insensitive and hence presumably unrelated to the Na/K transport system. The table does indicate that there is a twofold increase in Ca efflux with ATP (lines A and B), that there is a much larger increase in the effectiveness of Na_o and Ca_o in activating Ca

TABLE VII

A COMPARISON OF FLUXES IN AXONS WITH AND WITHOUT ATP

Property	ATP absent	ATP present	Reference
(A) Ca efflux into seawater $[Ca]_i = 0.3$ μM	0.11 pmol/cm²s	0.24 pmol/cm²s	DiPolo, 1974
(B) Ca efflux into Ca-free Tris seawater	0.006 pmol/cm²s	0.02 pmol/cm²s	DiPolo, 1974
(C) Na_o activation of Ca efflux	Half maximal at 150 mM Na_o	Much lower half maximum	Baker and Glitsch, 1973
(D) Ca_o activation of Ca efflux	Half maximal 2–3 mM	Half maximal 10–20 μM	Baker and Glitsch, 1973
(E) Ca-dependent Na efflux into seawater $[Na]_i = 80$ mM	None	20 pmol/cm²s	This study and Baker et al., 1969
(F) Ouabain-insensitive Na influx	20 pmol/cm²s	40 pmol/cm²s	Brinley and Mullins, 1967

efflux with ATP (lines C and D), and that Na efflux that is Ca_o dependent requires ATP (line E). Line F gives the magnitude of the ouabain-insensitive Na influx which is doubled by ATP. No appreciable fraction of this flux can be coupled to Ca efflux in normal axons since the increment in Na influx is ~20 pmol/cm²s and the increment in Ca efflux with ATP is of the order of 0.1 pmol/cm²s. However, in other tissues the transport of substances such as amino acids is known to be Na dependent and this ATP-dependent Na influx may be coupled to other transport reactions.

Effects of Mg_i on Ca Efflux

The systems responsible for transporting Mg and Ca outward in both squid axons and crustacean muscle fibers have properties in common which could be a result of the existence of a single carrier for both ions. For example, in both types of fiber, the divalent cation efflux is reduced by removal of external Na (Blaustein and Hodgkin, 1969; Baker and Crawford, 1972; Russell and Blaustein, 1974; Vogel and Brinley, 1973; Ashley and Ellory, 1972; Ashley et al., 1974). Divalent cation efflux is also reduced by an increase in Na_i (Baker et al., 1969; Blaustein et al., 1974; Vogel and Brinley, 1973). Moreover, divalent cation transport in both tissues seems to depend upon ATP in that the efflux from CN-treated fibers is increased by ATP (DiPolo, 1973; Baker and Crawford, 1972). Despite the similar energy and cation requirements for the two pumps, the present experiments indicate clearly that large changes in internal ionized Mg are without effect on the Ca pump. In fact, the collected data in Table IV provide no evidence of competition even when the concentration of ionized Mg exceeds that of Ca by a factor of 10^6. We infer that the selectivity of the Ca pump for Ca over Mg must be very high.

Effect of $[Na]_i$ on Ca Efflux

Internal Na produces a significant inhibition of calcium efflux. Two hypotheses are compatible with the experimental findings. In one, Na_i and Ca_i compete for binding to the same site, while in the other, an increase in Na_i decreases the energy in the gradient ($E_{Na} - E_m$). The concentrations of ions involved as competitors are so disparate (K_m for Ca_i = 0.3 μM, K_i for Na_i 40,000 μM) as to suggest that in fact there is no competition between Na_i and Ca_i. Further, if there were Na/Ca competition one would expect that as $[Ca]_i$ were increased, $[Na]_i$ would be a less effective inhibitor; however this is not observed. Thus, the present results fit better with the idea that Ca efflux depends primarily upon the quantity ($E_{Na} - E_m$), and that the effect of changing $[Na]_i$ is to alter the value of E_{Na}.

If external Na is removed and replaced by Li, the Na gradient upon which Ca^{++} extrusion depends is replaced by a Li gradient. In this case, the effec-

tiveness of Ca extrusion depends critically on the extent to which Li can replace Na in the system that moves Ca outward in exchange for a monovalent cation moving inward. Our experiments show that Ca efflux at low $[Ca]_i$ is not very dependent upon Na_o (i.e., that Li still produces a substantial efflux larger than the leak) and hence one infers that the ability of a Li gradient to extrude Ca is only slightly inferior to that of Na. In contrast, at high $[Ca]_i$, this is not the case: replacement of Na_o with Li_o leads to decreases in Ca efflux that approach 99% of the measured Ca efflux. The Na sensitivity of the Ca efflux in dialyzed axons may be somewhat less than in injected axons (e.g. Baker, 1972, Fig. 7; Blaustein and Hodgkin, 1969, Fig. 8), but in no case does removal of external Na reduce Ca efflux to the level required by the flux ratio equation.

Effect of Ca_i on Ca Efflux

The collected results shown in Fig. 8 relate Ca efflux to internal ionized Ca. The graph has two features of interest: (a) A measurable efflux of Ca (above chelator leak) exists at an ionized Ca as low as 0.005 μM. (b) The efflux is roughly proportional to Ca_i below 0.2–0.3 μM. The slope of the curve is much less above this concentration, although it does not saturate either on the basis of the current data or other studies in which internal ionized Ca was raised to very high levels (Blaustein et al., 1974; Mullins and Brinley, 1975). Moreover, a recent paper (Blaustein and Russell, 1975) indicates that Ca efflux is carrier mediated with complex kinetics at concentrations up to several hundred micromolar.

Our finding that Ca efflux in squid axons is roughly linear with [Ca] in the physiological range agrees with that of Caldwell (1972), who reached the same conclusion about Ca efflux in barnacle muscle fibers by comparing estimates of ionized Ca determined by the aequorin method (Ashley, 1970; Ashley and Ridgeway, 1970) with efflux of ^{45}Ca produced under similar conditions.

Fig. 8 allows an indirect estimate of the internal ionized [Ca] in intact squid axons by noting the $[Ca]_i$ at which the efflux and influx are equal. The mean influx in two intact axons measured during the present series of experiments was 0.17 pmol/cm²s, which is close to previously reported values of 0.08 (Hodgkin and Keynes, 1957) and 0.15 pmol/cm²s (Baker et al., 1969). From Fig. 8, equality of influx and efflux at 0.17 pmol/cm²s corresponds to $[Ca]_i$ of about 0.1 μM. However, these results are from ATP-free axons. If the effect of ATP (an approximate doubling of Ca efflux) is allowed for, correspondence would occur at a lower $[Ca]_i$, about 0.05 μM.

Influx and efflux in dialyzed fibers can also be compared to obtain estimates of Ca_i. In this case, Ca influx averaged 0.35 pmol/cm²s (Na_i 80 mM; ATP_i 5 mM). Adjusting the efflux curve in Fig. 8 for these internal con-

ditions requires an approximate doubling for ATP_i = 5 mM and approximate halving for Na_i = 80 mM (see Fig. 10). Thus, comparison of influx and efflux in dialyzed axons leads to a higher estimate of ionized Ca, about 0.2 μM. The conclusion from these indirect measurements, therefore, is that the internal ionized calcium may range between 0.05 and 0.2 μM. Baker et al. (1971), on the basis of aequorin luminescence in squid axoplasm, estimated the ionized Ca to be slightly higher, 0.3 μM.

In making this sort of comparison, it should be explicitly stated that this estimate of ionized [Ca] refers only to isolated squid axons bathed in an artificial seawater containing inorganic salts and may not apply *in situ*. Since the free Ca in squid hemolymph is only about 4 mM (Blaustein, 1974), Ca influx in axons in the living animal is probably lower.

There may also be very substantial differences between the behavior of the Ca fluxes in the living squid and that in the isolated, dialyzed axon. For example, because of the extremely low ionized Ca level in axoplasm, the time constant for change of internal free Ca is very short, probably in the order of tens of seconds. This means that were there no regulatory processes operating in the intact axon, changes in Ca influx or efflux such as might occur after excitation or increase in internal Na, would result in rapid, marked changes in ionized Ca near the membrane. In the dialyzed fiber such perturbations are presumably minimized by the presence of large amounts of chelator. However, in the intact axon such regulation could be exerted only by cytoplasmic proteins or mitochondria. Theoretical calculations by Baker et al. (1971, Appendix 1, Fig. 2) indicate that for reasonable increases in influx, mitochondria probably buffer the central axoplasmic core rather well, but allow the free Ca near the inner surface of the axoplasm to rise perhaps one or two orders of magnitude. Such large fluctuations in free Ca may well affect the permeability or other properties of the membrane in a manner different from that in dialyzed axons. For this reason conclusions about the transport system obtained from experiments on one preparation may not apply to the other.

Restoration of Internal Ionized Ca after Stimulation

Although the amount of Ca which enters a fiber during stimulation is small (Hodgkin and Keynes, 1957), there is nonetheless a significant increase in the concentration of internal ionized Ca. For example, the net influx of Ca produced by 600 impulse would nearly double the mean ionized Ca in a 500-μm fiber (assuming an initial ionized Ca of about 0.3 μM and a net influx of 0.006 pmol/cm² impulse). Ultimately, of course, this extra Ca must be extruded. If the extra Ca were uniformly distributed throughout the axoplasm, the relation between Ca efflux and ionized Ca reported in this paper would be qualitatively of the right sort to produce such extrusion since the efflux rises rather linearly with ionized Ca up to about 0.2–0.3 μM. However, the theoret-

ical analysis by Baker et al. cited above (1971) could be interpreted as indicating that after pulse changes of Ca_i at the surface, there is a persistent elevation of ionized calcium immediately adjacent to the membrane. Under these circumstances Ca extrusion by the axolemma would not be very sensitive to the level of ionized Ca.

In addition to the effect of Ca entry during stimulation upon internal ionized Ca, one must also consider the effects of Na entry. Since an increase in Na_i reduces Ca efflux (this paper; Blaustein and Russell, 1975) and increases Ca influx (Baker et al., 1969), the extra Na introduced by stimulation should affect Ca transport in the following two ways: (*a*) to oppose the extrusion of the extra Ca which has entered during stimulation, and (*b*) to favor net Ca entry during the poststimulation period, until such time as the Na pump has reduced the $[Na]_i$ to the prestimulation level. Both circumstances act so as to effect a temporary increase in Ca_i. Presumably short-term Ca homeostasis could be provided by the mitochondria and axoplasmic proteins, leaving long-term regulation to the transport mechanism in the membrane.

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