

# Sensitivity of Calcium Efflux from Squid Axons to Changes in Membrane Potential

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**ABSTRACT** Squid giant axons were internally dialyzed with a medium free of metabolic substrates but containing  $^{46}\text{Ca}$  buffered with EGTA to concentrations of free  $\text{Ca}^{++}$  in the range 0.01–230  $\mu\text{M}$ . At  $[\text{Ca}]_i$  of 1.0  $\mu\text{M}$  or greater, Ca efflux was in the range of 1–3 pmol/cm<sup>2</sup> s, was dependent on  $[\text{Na}]_o$  and  $[\text{Ca}]_o$ , and was sensitive to membrane potential. At lower  $[\text{Ca}]_i$ , the sensitivity of Ca efflux to membrane potential was greater. Hyperpolarization of the membrane increased, and depolarization decreased Ca efflux over the range of potentials studied (–20 to –100 mV). The maximum sensitivity of Ca efflux to membrane potential was of the order of an  $\epsilon$ -fold increase in Ca efflux for a 25-mV increase in  $E_m$ ; this sensitivity of Ca efflux to membrane potential was lost if  $[\text{Na}]_o$  was removed and was greatly reduced when  $[\text{Ca}]_i$  was increased to 230  $\mu\text{M}$ .

## INTRODUCTION

Calcium efflux from squid giant axons is known to depend upon the presence of  $[\text{Na}]_o$  in seawater and on the metabolic state of the axon, since efflux is increased about fivefold by poisoning the axon with CN (Blaustein and Hodgkin, 1969). While the content of Ca in nerve is of the order of 0.5 mmol/kg water, there is compelling evidence that only a minute fraction of this  $[\text{Ca}]$  is ionized (Baker et al., 1971). Since Ca forms poorly dissociated complexes with a variety of intracellular compounds, and since mitochondria are known to accumulate this ion, a formidable array of difficulties is presented to the investigator wishing to examine the Ca fluxes in a nerve fiber.

Internal dialysis is a technique that appears quite suited to flux measurement under the circumstances detailed above. It is capable of removing substrates from axoplasm, thus preventing metabolically dependent shifts of Ca between intracellular compartments, and it can supply labeled Ca buffered to any desired concentration of free Ca with EGTA or other such substances. Since free  $[\text{Ca}]$  is unknown, but may well be in the range of  $10^{-6}$  to  $10^{-7}$  M, it

is necessary to buffer fluids quite strongly with EGTA in view of both the large quantity of Ca known to be present inside a fiber and the fact that reagents used to prepare internal dialysis fluids can be expected to be contaminated with appreciable quantities of free Ca. It has been shown (DiPolo, 1973) that the Na/Ca exchange system in squid axons does not require ATP in order to function; further study (DiPolo, 1974) however, has shown that ATP does increase Ca efflux. In most of the experiments to be reported, no substrate was included in the dialysis fluid, in order to study the Na/Ca system in its simplest mode of operation.

The main purpose of the experiments to be reported was to examine the dependence of Ca efflux on membrane potential. Such a dependence is to be expected if the Na/Ca coupling mechanism operates at low levels of ionized calcium, as the following calculations indicate. Let  $^{Ca}m_o$  represent the calcium efflux coupled to sodium influx ( $^{Na}m_i$ ). If the energy for this component comes from the sodium gradient then:

$$^{Na}m_i(E_{Na} - E_m) \geq 2^{Ca}m_o(E_{Ca} - E_m),$$

or

$$\frac{^{Na}m_i}{2^{Ca}m_o} \geq \frac{(E_{Ca} - E_m)}{(E_{Na} - E_m)}.$$

For  $Ca_o = 10$  mM and  $Ca_i = 0.1$   $\mu$ M, the coupling ratio is at least 4, implying that the carrier transfers net charge during the cycle and hence is electrogenic, although in a sense opposite to that used for Na pumping. The term "carrier" is used here to denote any mechanism that allows a coupling of the Na fluxes to the Ca fluxes. Electrogenic is defined as any coupling mechanism operating in a mode such that unequal quantities of electric charge are transferred per cycle of the coupling mechanism.

## METHODS

### *Experimental Material*

The experiments reported here were performed upon live specimens of *Loligo pealei* during May and June 1973 at the Marine Biological Laboratory in Woods Hole, Massachusetts.

### *Dialysis Technique*

The basic technique is as described previously (Brinley and Mullins, 1967), with the substitution of a plastic dialysis capillary for the glass tubes originally used. The properties of this material have been described elsewhere (Brinley and Mullins, 1974). Procedures for evaluating dialysis capillaries have been previously reported (Brinley and Mullins, 1967).

In order to alter the potential across the axolemma, a 6–25- $\mu\text{m}$  platinum-iridium wire was placed in the lumen of the capillary and connected via a 7-M $\Omega$  current-limiting resistor to a current source. The transmembrane potential was altered by manually adjusting the potential applied to the resistor and was measured directly by a KCl-filled electrode inserted longitudinally into the center of the dialyzed area. This arrangement is shown in Fig. 1.

Although the lines of current flow are primarily radial in the center of the porous regions and hence result in nearly uniform transmembrane potential, there will be a significant longitudinal vector in the end regions. Furthermore, the cable properties

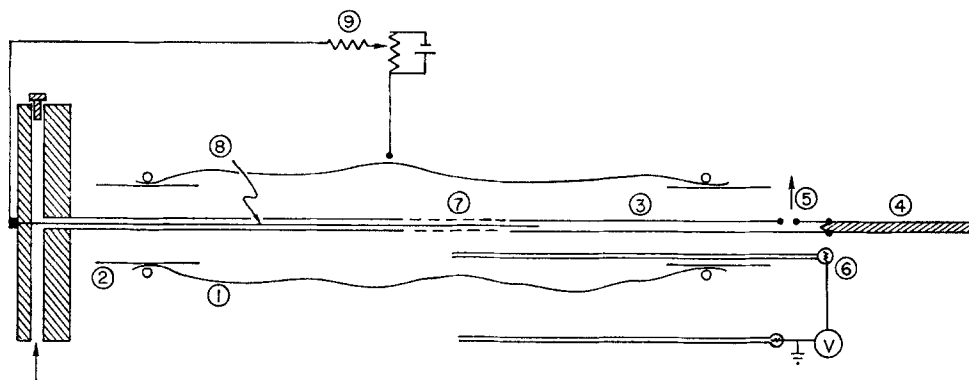


FIGURE 1. A diagram of the experimental arrangement for dialysis and for membrane potential control. The squid axon (1), tied to glass cannulae (2) at both ends, is impaled with a plastic tube (3) by drawing a tungsten wire (4) through the axon and thus guiding the plastic tube to its location. A small hole (5) allows the escape of dialysis fluid that is continually moving through the tube. A glass capillary and calomel electrode (6) monitor membrane potential in the porous region (7) of the dialysis tube, while a Pt-Ir wire (8) inside the porous capillary delivers current to the axoplasm from a constant current generator (9).

of the axon cause significant variations in the transmembrane potential as measured along the length of the fiber. No special effort was made to compensate for the longitudinal variation of potential outside of the porous regions because the only isotopic flux measured in our experiments was that from the central 60–80% of the porous regions. (See Fig. 6, Brinley and Mullins, 1967). In this region direct measurement of the membrane potential with the internal KCl pipette indicated that the potential is constant to within 5 mV for hyperpolarizing currents. The apparatus is inadequate for significant depolarizations because the space constant becomes too small to permit a uniform distribution of potential without some sort of a guard system. To examine the effects of depolarizations upon the calcium fluxes, the fiber was depolarized with high external potassium.

Since the flexibility of the plastic tubing precluded pushing it directly through the axoplasm, a 100- $\mu\text{m}$  tungsten wire was cemented to the end of the plastic tubing. This wire was guided through the axon under microscopic control. After the leading edge of the tungsten wire had emerged from the axon, it was attached to a manipu-

lator and the plastic tubing was pulled into position. The tungsten wire and all fluid-filled portions of the dialysis assembly were carefully isolated from ground to avoid short circuiting the interior of the fiber.

### Solutions

The solutions used in this study are listed in Table I. The external solutions are conventional. The composition of the internal solution, originally chosen to approximate the composition of squid axoplasm as given by Deffner (1961), has undergone repeated revision as it has become obvious that the electrical and transport properties

TABLE I  
COMPOSITION OF SOLUTIONS USED

Ion	External solutions				Internal solutions		
	ASW	Na-free ASW	Ca-free ASW	100 K ASW	AE	AF	CO
	<i>mM</i>						
K	9	9	9	100	309	389	308
Na	429		429	329	80		40
Li		429					
Mg	48	48	57	48	4	4	
Ca	9	9		9			
Cl	496	496	496	487	88	88	
Isethionate					151	151	
D-Aspartate					151	151	342
CN	1	1	1	1	1	1	1
SO <sub>4</sub>	25	25	25	25			
EDTA	0.1	0.1	0.1	0.1			
TES	4	4	4	4	5	5	5
Taurine					275	275	275
pH	7.5	7.5	7.5	7.5	7.0	7.0	7.0

Note: ASW = artificial seawater.

of the axolemma are not critically dependent upon the exact composition of the internal solution.

The composition of the internal solutions given in Table I does not include any calcium or EGTA. These were added as needed. The concentrations actually used are given in the appropriate figure or table. In calculating the ionized calcium concentration, the apparent stability constant for CaEGTA at pH 7.0 was taken as  $5 \times 10^{-6} \text{ M}^{-1}$ , using the stability constants given by Bjerrum et al. (1957). Actual ionized [Ca] depends on pH, temperature, and the ionic strength of the solution. Because the solutions used for dialysis differ from those cited above, ionized [Ca] values are nominal. Solutions contained 5 mM KTES (TES: *N*-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid) to minimize pH changes. The choice of the calcium-buffering system for these experiments posed several problems. The axoplasm contains anions capable of some calcium binding as well as about 400  $\mu\text{mol/kg}$  of calcium which is isotopically exchangeable but poorly dialyzable. Both of these considerations,

as well as the possibility that reagents contain some  $\text{Ca}^{++}$  as a contaminant, dictate that the buffering system be present in fairly high concentration ( $\sim\text{mM}$ ) in order to achieve control of ionized Ca. On the other hand, the presence of a large concentration of CaEGTA raises the possibility that one may have an efflux of radioactive calcium either as  $\text{Ca}^{++}$  or as Ca in some bound or chemically complexed form. In practice the dialysis solution contained CaEGTA in a concentration of about 1–2 mM. Evidence that significant efflux of  $^{46}\text{Ca}$  in a chelated form did occur is considered in the Results section.

#### *Dialysis Chamber.*

The details of the chamber and its operation are essentially as described earlier (Brinley and Mullins, 1967).

#### *Tracer Methodology*

Radioactive  $^{46}\text{Ca}$  was obtained as the chloride either from the General Electric Company (Pleasanton, Calif.) or New England Nuclear, (Boston, Mass.). Additions of this material were combined with cold  $\text{CaCl}_2$  and EGTA to give dialysis solutions with the desired Ca and EGTA composition. Measurements of calcium efflux were made by dialyzing with radioactive solutions and collecting the isotope emerging from the axon while it was immersed in a continuous flow system. Samples were collected at 1- or 2-min intervals by means of an automatic fraction collector.

The samples were dried in an oven and counted in a low level gas flow counter with an anticoincidence guard. Appropriate dilutions of the dialysate for standards were added to seawater and handled in the same manner.

Efflux, as picomoles per square centimeter per second, was calculated from the counts recovered in the external solution, the internal specific activity, and the area of axon in the collecting portion of the chamber.

## RESULTS

### *Ca Efflux under $\text{Ca}_o$ -Free and $\text{Na}_o$ -Free Conditions*

All axons were pretreated with 1 mM CN seawater in order to aid in the removal of both ATP and Ca from mitochondria. They were then predialyzed for 30–60 min with either CaEGTA at the concentration of Ca that it was proposed to use during the experiment, or with 1 mM MgEGTA alone. The internal dialysis fluid was then changed to one containing  $^{46}\text{Ca}$  buffered to the desired Ca concentration and efflux measurements were begun. The notation  $[\text{Ca}]_i$  will be used for nominal free or ionized  $[\text{Ca}]$  in axoplasm.

We have used EGTA/CaEGTA mixtures at a concentration of about 1 mM; this means that from  $10^3$  to  $10^4$  more Ca is in the form of CaEGTA than is in  $\text{Ca}^{++}$ . In turn, one must then be concerned about the possible leaks of CaEGTA from the fiber since  $^{46}\text{Ca}$  coming from this source will contribute to the efflux. One way of checking this point is to measure the Ca efflux in fibers with very low  $[\text{Ca}]_i$ . Under these conditions, one might expect that the leak of buffer would be a larger fraction of total efflux.

The squid axon, while undoubtedly slightly permeable to large anions and neutral molecules, also has imperfections in its membrane that, from the view of voltage clamping, are lumped as "leakage currents." There are reasons for believing that leakage pathways allow a totally nonselective diffusion of solutes of all sorts across the membrane (Mullins, 1966) and that one must expect that Ca buffer will be no exception.

An experiment to check on the possibility that Ca efflux and membrane leakage are related is shown in Fig. 2. Free  $\text{Ca}^{++}$  was adjusted to a very low

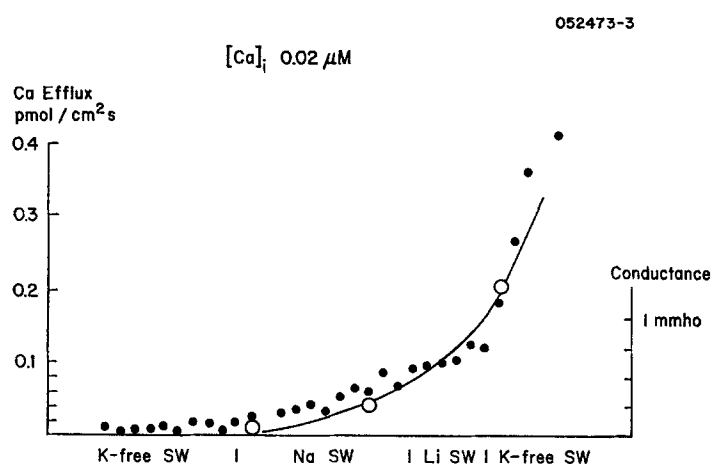


FIGURE 2. Ca efflux is plotted as a function of time (each point represents 2 min) for an axon dialyzed with an ATP-free medium (AE). Solid points are Ca efflux while open circles are membrane conductance measured at  $E_m = -70$  mV. Values for conductance per square centimeter of membrane surface can be obtained by multiplying the values shown by 3.27 to convert from the surface area of the capillary controlling the membrane dialysis to 1  $\text{cm}^2$  of membrane surface.

value ( $0.02 \mu\text{M}$ )<sup>1</sup> in a fiber which initially had a very high membrane resistance ( $\sim 5 \text{ K}\Omega\text{cm}^2$ ). Resistance and flux measurements were followed with time and it is apparent that Ca efflux and membrane conductance follow similar time courses. Most of the axons used in the work to be reported here had membrane resistances in the range of 700–1,000  $\Omega\text{cm}^2$  and this would correspond to a leakage Ca efflux of  $\sim 0.05 \text{ pmol/cm}^2\text{s}$ . We conclude, therefore, that in the absence of  $[\text{Na}]_o$  and  $[\text{Ca}]_o$ , the residual Ca efflux (in the absence of substrate) may be a leakage of Ca buffer.

By an extension of this reasoning, the rather low Ca efflux observed when the seawater bathing the axon is Ca- and Na-free might also be supposed

<sup>1</sup> Such concentrations are at the limits of the buffering range of CaEGTA/EGTA mixtures. The  $[\text{Ca}]_i$  is in the range 0.01–0.03  $\mu\text{M}$ .

to be a leak of the Ca buffer if Ca efflux depends totally on the presence of either external Ca or Na. Table II shows that while at low  $[Ca]_i$  (in the range of 0.01–0.07  $\mu M$   $Ca_i$ ) Ca efflux is rather variable, an axon with a free  $Ca_i$  of 200  $\mu M$  had a value for Ca efflux of the same magnitude under Ca-free, Na-free conditions; it had a Ca efflux of almost 10 times greater magnitude in the presence of these external cations. Further information from Table II shows that while the measured membrane conductance of the fibers

TABLE II  
RELATIONSHIP BETWEEN  $Ca$  EFFLUX AND MEMBRANE CONDUCTANCE

Axon	$[Ca]_i$	Ca efflux	Dialysis time	$g_m$	Ca efflux/ $g_m$
	$\mu M$	$pmol/cm^2s$	$min$	$mmho/cm^2$	$(fmol/cm^2s)/(mmho/cm^2)$
052173-1	0.01	0.06	85	1.5	40
	0.01	0.21	107	6.5	32
052273-3	0.07	0.05	117	1.2	41
	0.07	1.3	141	13.7	95
052373-1	0.01	0.10	78	1.4	64
052473-1	0.01	0.011	46	0.16	61
	0.01	0.056	64	1.3	43
	0.01	0.22	86	4.1	54
060673-3	200.	0.16*	146	3.3	50
				Mean	53

The membrane conductance was measured by the increment in current required to displace the membrane potential from  $-55$  to  $-70$  mV. All measurements were in seawater except as noted below.

\* Efflux into Ca-free, Na-free seawater. Efflux into Na + Ca seawater was 1.2  $pmol/cm^2s$ .

studied varied from 0.16–13.7  $mmho/cm^2$ , the ratio of Ca efflux (femtomoles per square centimeter per second) divided by membrane conductance (millimhos per square centimeter) had a mean value of about 50 and a variation of substantially less than a factor of 2.

In some circumstances, the efflux appears more related to  $[CaEGTA]_i$  than  $[Ca]_i$ . Line 13 of Table III shows an axon (051673-1) which was treated first with a CaEGTA buffer at a concentration of 44 mM, followed by a change to one of 0.8 mM. The change of ionized Ca in the two solutions was very small (from 0.13 to 0.17  $\mu M$ ), yet the apparent Ca efflux (6.3  $pmol/cm^2s$  vs. 0.35  $pmol/cm^2s$  for the 44 mM vs. 0.8 mM CaEGTA) was almost 20 times larger with the high CaEGTA solution.

Some preliminary experiments (Mullins et al., 1975) show that when  $^{14}C$ -EDTA is equilibrated with axoplasm by dialysis at a concentration of 1.25

TABLE III  
CALCIUM EFFLUX IN DIALYZED SQUID AXONS (15–17°C)

Axon number	Axon diameter	[Na] <sub>i</sub>	Internal [Ca]		-E <sub>m</sub>	Ca efflux				Flux with hyperpolarization			
			Total	Ionized		Sea-water	Ca-free	Na- and Ca-free	Na-free	-ΔE <sub>m</sub>	Ca efflux	Increase in Na + Ca-sensitive Ca efflux	
	μm	mM	mM	μM	mV	pmol/cm <sup>2</sup> s				mV	pmol/cm <sup>2</sup> s	n-fold/25 mV ΔE <sub>i</sub>	
052173-1	810	40	1.4	0.01	45	0.06				0.06			
052373-1	750	40	1.4	0.01	56	0.11					55	0.23	(1.5)*
052373-2	756	40	1.4	0.01		0.10							
052373-3	675	40	1.4	0.01		0.09				0.09			
052473-1	810	40	0.8	0.01	53	0.01					17	0.02	(2.9)
052473-2	800	40	0.8	0.01	50	0.10							
060473-1	750	100	2.8	0.05	51	0.30			0.40		25	0.85	(2.8)
060473-2	800	100	2.8	0.05	52	1.0	0.30	0.30			16	1.4	1.9
052273-1	675	0	0.8	0.07	53	0.05							
052273-2	700	0	0.8	0.07	60	0.02			0.02				
052273-3	756	0	0.8	0.07	57	0.05				0.05			
052073-1	700	0	0.8	0.07	55	0.09			0.09		35	0.09	—
051673-1	675	20	44	0.13	63	6.3	4.8		3.5				
		40	0.8	0.17	57	0.35			0.15				
051673-2	756	40	0.8	0.17		0.55				0.15			
050773-2	756	40	0.8	0.50	54	0.36	0.14		0.05		10	0.45	(3.1)
051073	648	80	1.8	0.50	52	0.19	0.14	0.015			~5	0.20	—
051073-2	594	80	0.8	0.50	58	0.26		0.16			10	0.39	4.3
051273-2	775	80	1.8	0.50	28	0.38					20	0.75	(2.2)
051273-3	700	80	1.8	0.50	40	0.96		0.32			18	1.0	1.1
051373-1	621	80	1.8	0.50	39	0.80		0.08			18	1.1	1.6
051373-2	729	80	1.8	0.50	~50	0.20		0.035			37	0.25	1.2
051473-2	783	80	1.8	0.50	52	0.20		0.038		0.14			
060573-1	700	100	1.0	230.	~50	0.9		0.03			32	1.2	1.3
060573-2	650	100	1.0	230.	~50	1.0		0.009			39	1.2	1.1
060673-1	750	100	1.0	230.	~50	3.0		0.09			35	3.9	1.2
060673-2	804	100	1.0	230.	~50	2.7	0.06	0.065					
060673-3	750	100	1.0	230.	56	1.4	0.16	0.03			25	1.8	1.3

\* Values in parentheses not corrected for leakage.

mM, the efflux of EDTA is 0.032 pmol/cm<sup>2</sup>s and this efflux is insensitive to membrane potential. The value is not greatly different from our estimate (0.05 pmol/cm<sup>2</sup>s) for EGTA efflux in this present study.

An important point that requires emphasis is that in the absence of Na<sub>o</sub> and Ca<sub>o</sub> we have never observed a sensitivity of Ca efflux to membrane potential. We conclude, therefore, that the mechanism that transfers CaEGTA across the membrane is electroneutral.

#### Effect of [Na]<sub>o</sub> on Ca Efflux

At the very lowest [Ca]<sub>i</sub> studied (0.01 μM), Na-free seawater had no effect on Ca efflux as would be expected if, as noted earlier, efflux by Ca/Na exchange is very much smaller than the leak of the buffer. At a [Ca]<sub>i</sub> of 0.5



$\mu\text{M}$ , and of  $0.17 \mu\text{M}$  (single axons for each concentration), there was an easily measurable reduction of Ca efflux when Na-free conditions were imposed. However, at both concentrations there was a further threefold drop in Ca efflux when  $\text{Ca}_o$ -free as well as Na-free conditions were imposed. Our experience with Na-free conditions (five axons) can be summarized by saying that with the exception of when  $[\text{Ca}]_i$  is  $0.01$ – $0.07 \mu\text{M}$ , Na-free conditions reduce Ca efflux less than do Ca-free, Na-free conditions (see Table III). There is too much variation from axon to axon to make any quantitative statement regarding this result.

*Analysis of the Effects of Sodium- and Calcium-free Seawater on Ca Efflux*

The argument has been made that experiments using Na-free, Ca-free seawater measure the leak of CaEGTA buffer from the axon rather than the efflux of ionized Ca. Since the concentration of CaEGTA in the experiments reported was in the range of  $1 \text{ mM}$  (with a single exception) one would expect that at low  $[\text{Ca}]_i$ , Ca efflux would be insensitive to Na-free, Ca-free conditions, while at high  $[\text{Ca}]_i$ , virtually all Ca efflux would be sensitive. Fig. 3 is a plot of the data of Table III (for 18 axons) giving the percent of the observed Ca efflux in seawater that remains when Na- and Ca-free conditions were imposed. From the plot it is clear that at low  $[\text{Ca}]_i$ , virtually 100% of the Ca efflux is insensitive to Na and Ca while at  $230 \mu\text{M}$  less than 2% of the efflux remains under the same conditions.

The response of Ca efflux at  $[\text{Ca}]_i < 0.2 \mu\text{M}$  to Na-free and Ca-free condi-

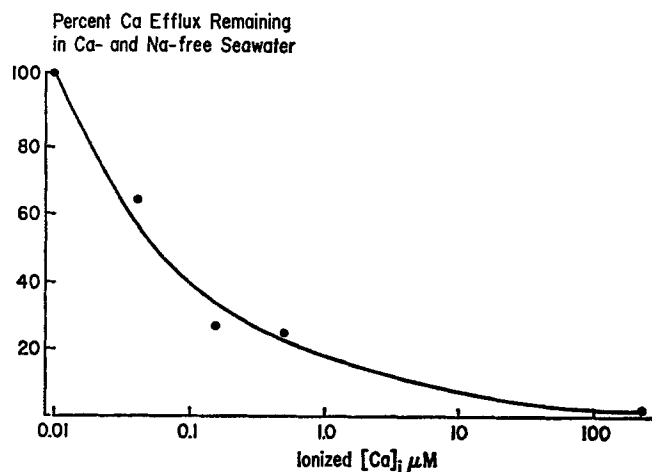


FIGURE 3. The data from Table III are plotted to show the mean value of Ca efflux that remains when an axon is transferred from seawater to Ca- and Na-free seawater as a function of ionized  $[\text{Ca}]_i$  (semilogarithmic scale). Efflux is expressed as percent of control efflux remaining in Ca- and Na-free seawater.

tions has not been studied in detail sufficient to be certain that all or most of the efflux is buffer leak. In particular, since the principal cation in Na-free seawater is  $\text{Li}^+$ , the extent to which  $\text{Li}^+$  can energize Ca extrusion remains unknown.

#### *Effect of Membrane Potential on Ca Efflux*

If a Ca extrusion mechanism is to extract energy from the Na concentration gradient and use this to balance Ca influx, there are several points of experimental interest that such a scheme would entail. First, the question is whether there is to be an obligatory coupling of a fixed number of  $\text{Na}^+$  to  $\text{Ca}^{++}$ .

The coupling ratio of Na moving inward to Ca moving outward when  $E_{\text{Na}} = +40$  mV,  $E_m = -60$  mV,  $[\text{Ca}]_o = 6.5$  mM<sup>2</sup> is shown below (assuming 100% efficiency for the exchange process):

Na/Ca ratio	$[\text{Ca}]_i$
	$\mu\text{M}$
4.6	0.01
4.0	0.10
3.6	0.50
3.4	1.00
3.0	8.00
2.0	320.00

From an energetic point of view, at a  $[\text{Ca}]_i$  of 200  $\mu\text{M}$ , a nearly electro-neutral transfer of 2 Na in for 1 Ca out is possible (at 100% efficiency) assuming a  $[\text{Ca}]_o$  of 6.5 mM. A fixed coupling of 3 Na in per Ca out would lead to a  $[\text{Ca}]_i$  of 10  $\mu\text{M}$ . Clearly, lower values of  $[\text{Ca}]_i$  require either a higher than 3 Na/1 Ca ratio or a lower  $[\text{Ca}]_o$  than that of seawater. The actual activity of  $\text{Ca}^{++}$  in squid blood has been estimated to be of the order of 1 mM (Blaustein, 1974). Experimental studies on the squid axon, however, invariably use seawater as the external fluid and it seems likely that useful flux values will only be obtained on isolated axons. Thus, the following experimental results are related to isolated axons with internally buffered [Ca].

In Fig. 4 is shown the efflux from an axon with an ionized [Ca] of 0.07  $\mu\text{M}$ . This flux was highly stable for over 180 min and was totally unaffected by Na-free, Ca-free seawater or by a change in membrane potential from  $-55$  to  $-90$  mV. We have suggested earlier that this behavior is to be ex-

<sup>2</sup> Estimated as free [Ca] in seawater containing 25 mM  $\text{SO}_4$ , 48 mM Mg, 9 mM Ca, and assuming  $\text{MgSO}_4$  and  $\text{CaSO}_4$  dissociation constants are both  $5 \times 10^{-3}$  M.

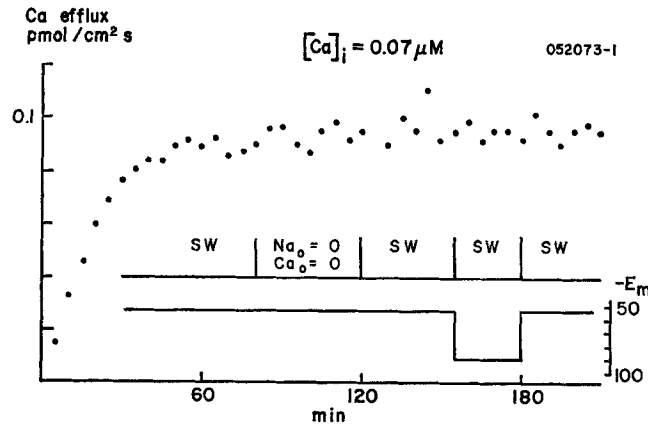


FIGURE 4. Ca efflux is shown as a function of time for an axon where solution changes were made as indicated on the legend. Membrane potential was  $-55$  throughout except in the one period where it was held at  $-90$  mV.

pected if a major fraction of the Ca efflux is really a leak of CaEGTA rather than one of ionized Ca. This axon had an unusually high membrane conductance of  $2.6$  mmho/cm<sup>2</sup> which would, according to our estimates, mean that virtually all of the  $0.09$  pmol/cm<sup>2</sup>s of efflux was buffer leak. Two other axons at  $[Ca]_i = 0.01$ , two at  $[Ca]_i = 0.05$ , and one additional axon at  $[Ca]_i = 0.07$  displayed a similar lack of sensitivity to Na-free and/or Ca-free, Na-free seawater.

Most axons with a low  $[Ca]_i$  did not behave in the way that of Fig. 4 did however, and Fig. 5 shows an axon with a  $[Ca]_i$  of  $0.01$   $\mu$ M. This axon had an efflux similar to that in Fig. 4; this efflux declined abruptly when  $100$  mM  $K^+$  seawater was applied to the axon. Hyperpolarization sufficient to restore the resting membrane potential also returned the Ca efflux to normal. A return to seawater gave a return to a normal efflux and a hyperpolarization of the membrane increased Ca efflux. Unfortunately Na-, Ca-free seawater was not applied to the axon so that an evaluation of buffer leak by this method is not possible. Electrical measurement of membrane conductance yielded a value of  $1.4$  mmho/cm<sup>2</sup> which would be equivalent to a buffer leak of  $\sim 0.05$  pmol/cm<sup>2</sup>s. In all, 16 axons were examined for a response to hyperpolarization and 15 of these gave a measureable increase in Ca efflux. The single axon that did not respond had a total insensitivity to Na-free, Ca-free conditions; hence we conclude that its efflux was largely buffer leak.

In calculating the effect of hyperpolarization on Ca efflux it seems reasonable to use the following procedure. The Ca efflux at the normal resting potential of the membrane is considered to comprise a Na + Ca-dependent fraction  $r$  plus a potential insensitive component  $l$ , either wholly or partially

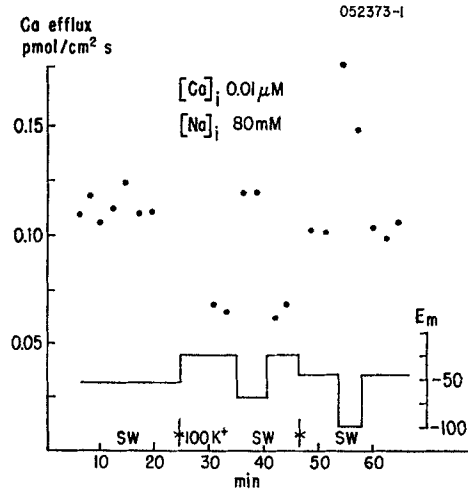


FIGURE 5. Ca efflux is shown as a function of time for an axon in either seawater or 100 K seawater. Membrane potential changes induced either by the solution applied or by external electric currents are shown at the bottom of the chart.

buffer leak. Ca efflux is therefore  $(r + l)$ . At a hyperpolarized membrane potential, Ca efflux is considered to be  $(r + l + i)$  where  $i$  is the increment in Ca efflux produced by the change in membrane potential. Since various magnitudes of membrane potential change were employed, it is necessary to adjust  $i$  by dividing by  $\Delta EF/RT$  (where  $\Delta E$  is the change in membrane potential) so that alterations in efflux can be normalized to a 25-mV change in membrane potential. The final result is expressed as hyperpolarized/resting Ca efflux  $[r + i(25 \text{ mV})]/r$ .

As an example of how the analysis of potential effects is made, the data from the curve shown as Fig. 6 is set forth below.

Resting Ca efflux (at  $t = 20$  min):

$$(r + l) = 0.9 \text{ pmol/cm}^2\text{s.}$$

Leakage Ca efflux ( $t = 40\text{--}60$  min):

$$l = 0.1 \text{ pmol/cm}^2\text{s.}$$

Hyperpolarized Ca efflux (point marked 18 mV and subsequent point):

$$r + l + ai = 1.35 \text{ pmol/cm}^2\text{s,}$$

$$r + l + bi = 1.10 \text{ pmol/cm}^2\text{s,}$$

(where  $a + b = 1$ ) and represent fractional collections of the increment in Ca efflux:

$$2(r + l) + i = 2.45$$

## Subtract

$$2(r + l) = 1.80$$

$$i = 0.65.$$

As  $\Delta E$  was 18 mV

$$i/(\Delta EF/RT) = 0.65/(18/25) = 0.90$$

$$[r + i(25 \text{ mV})]/r = (0.8 + 0.9)/0.8 = 2.1.$$

Thus hyperpolarization increased Ca efflux 2.1-fold for a 25-mV increase in membrane potential in this experiment. With some axons, Ca efflux under Na- and Ca-free conditions was not measured, hence only the ratio  $(r + l + i)/(r + l)$  is available. These ratios are shown in parentheses in the last column of Table III. Such values would be substantially larger if (a) the flux measured were small and (b) the leakage were likely to be a substantial fraction of the measured Ca efflux.

One might question whether the effects of hyperpolarization that are observed are to be related to the membrane potential per se or to the Na gradient ( $E_{\text{Na}} - E$ ). In this connection, most of the experiments were done at  $[\text{Na}]_i = 40, 80, \text{ or } 100 \text{ mM}$ , corresponding to ( $E_{\text{Na}} - E$ ) of 120, 102, and 97 mV. A 25-mV increment in membrane potential thus would change ( $E_{\text{Na}} - E$ )

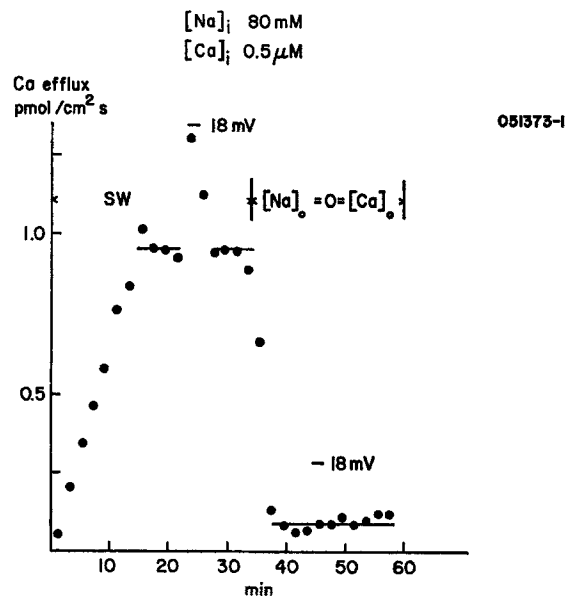


FIGURE 6. Ca efflux is shown as a function of time. In seawater, an 18-mV hyperpolarization for 2 min increased Ca efflux. When  $[\text{Na}]_o$  and  $[\text{Ca}]_o$  were both decreased to zero, a similar hyperpolarization was without effect on Ca efflux.

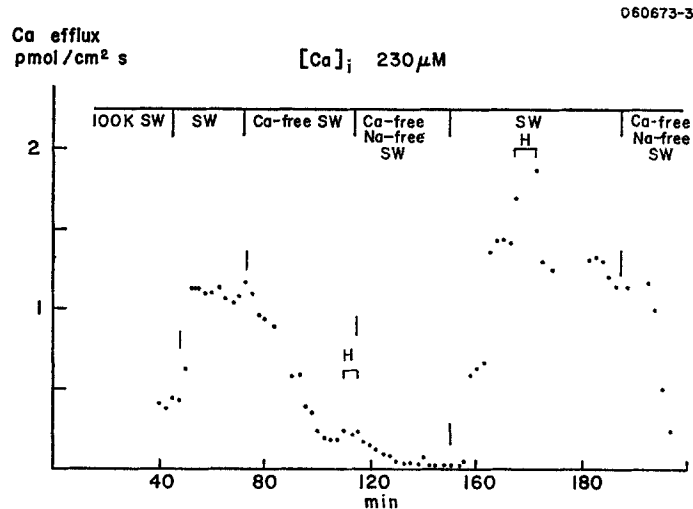


FIGURE 7. Ca efflux as a function of time is shown for an axon with the indicated  $[Ca]_i$ . Hyperpolarizing pulses in Ca-free seawater and in seawater are shown in brackets with the label "H." They were 25 mV more negative than the membrane potential ( $-55$  mV).

by about 25%, while it changes Ca efflux by greater than  $e$ -fold in a number of the axons examined.

In contrast with these findings at low  $[Ca]_i$ , when the admittedly unphysiological  $[Ca]_i$  of  $230 \mu\text{M}$  is used, the experimental results are quite different. Fig. 7 shows the efflux of Ca from an axon with this high  $[Ca]_i$  as a function of time. The axon was in 100 K seawater at the start. It had a low efflux which rose when normal seawater replaced the high  $K^+$  seawater and it fell to 20% of its value when Ca-free seawater was applied. Subsequent treatment with Na-, Ca-free seawater caused a further decline in Ca efflux to a level less than 2% of the value in normal seawater. The efflux returned to control values in seawater, was sensitive to hyperpolarization, and declined once again in Na- and Ca-free seawater. There was a very small absolute effect of hyperpolarization in Ca-free seawater, although the fractional sensitivity of the Ca efflux ( $n$ -fold/25 mV) was 1.3 both in seawater and in Ca-free seawater.

Systematic information regarding the effects of potential on Ca efflux is somewhat fragmentary but the values shown in Table III indicate that at low  $[Ca]_i$  the increment in flux approaches or exceeds an  $e$ -fold increase in Ca efflux per 25 mV of hyperpolarization, while at  $[Ca]_i = 230 \mu\text{M}$ , the values are substantially less than this. For a  $[Ca]_i$  of  $0.01$ – $0.05 \mu\text{M}$  the mean value of the increment in flux with hyperpolarization is 2.2; the same value is obtained for  $[Ca]_i$   $0.01$ – $0.5 \mu\text{M}$ , while for  $[Ca]_i$   $230 \mu\text{M}$  the increment is only 1.2. The data in the last three columns of Table III give the hyper-

polarization applied, the Ca efflux occurring during the period of hyperpolarization, and a calculation of the increment in Ca efflux expressed as the number of fold the efflux increases per 25 mV of hyperpolarization. The efflux considered is the Na- and Ca-dependent Ca efflux in order that the flux be corrected for buffer leak. Values uncorrected for leak are shown in parentheses.

A tentative conclusion from these results is that the Ca/Na exchange at high  $[Ca]_i$  is close to an electroneutral one, while at low  $[Ca]_i$  it is not. Values for Ca efflux with hyperpolarization are relatively independent of leakage at high  $[Ca]_i$ ; so that they can be considered the most reliable.

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

The data from Table III showing Ca efflux into seawater as a function of  $[Ca]_i$  are plotted in Fig. 8. Although there is considerable scatter in the data, one can infer that Ca efflux is a nonlinear function of  $[Ca]_i$  at values greater than 1  $\mu M$  when the scatter is least. At a  $[Ca]_i$  of 0.01  $\mu M$  all values that we have show that Ca efflux is independent of  $Na_i$ , so that if leakage corrections had been made, efflux would have been very small.

At higher levels of  $[Ca]_i$  our values agree with those of DiPolo (1973); an extension of these measurements to lower  $[Ca]_i$  shows that at a  $[Ca]_i$  of 1.0  $\mu M$ , Ca efflux can be expected to be  $\sim 1$  pmol/cm<sup>2</sup>s with most of this efflux Na and Ca dependent.

#### *Effect of ATP on Ca Efflux*

All of the experiments reported so far have been conducted in the absence of metabolizable substrates. As the results of DiPolo (1973) have shown, the

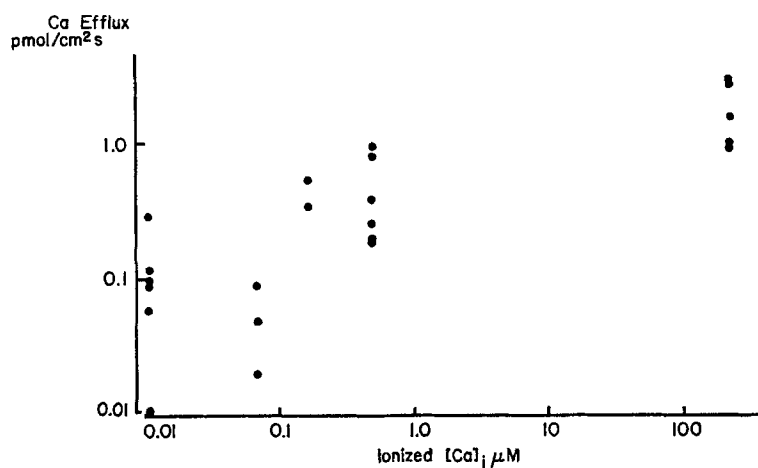


FIGURE 8. The data of Table III giving Ca efflux into Na + Ca containing seawater as a function of  $[Ca]_i$  are shown on a double logarithmic plot.

Na/Ca exchange mechanism can operate apparently normally in the absence of ATP. In a normal axon, however, there is an ATP concentration of 3–4 mM so that a more physiologically relevant system would be one where ATP was included in the dialysis system. This was not done for most of the experiments we performed, mainly because ATP can be expected to cause shifts of Ca into mitochondria and in other ways make the behavior of the Ca/Na exchange system more complicated to analyze. Nevertheless, ATP does have a prominent effect on Ca efflux as the results of a single experiment shown in Fig. 9 indicate. The axon was dialyzed with the usual, substrate-free internal fluid with a CaEGTA/Ca buffer to fix the ionized Ca at  $0.5 \mu\text{M}$ . After obtaining a base line and a response to a 10-mV hyperpolarizing pulse, the seawater was made Ca free and a decline in efflux to about 0.4 of normal was observed. After a return to normal seawater, ATP 5 mM, ArgP 5 mM was added to the dialysis fluid and the experiment continued. In this experiment the ATP was present as MgATP. The addition of MgATP and MgPA provided some free Mg to the axoplasm which displaced Ca from the EGTA and thus increased free Ca. However, calculation of the equilibrium concentration of all moieties under these conditions (S. G. Spangler, personal communication) shows that the free Ca will rise by less than 15%. We conclude, therefore, that the nearly threefold increase in calcium efflux seen in this experiment cannot be the result of a large change in ionized calcium concentration occasioned by the presence of unchelated magnesium. Ca efflux rose slowly with addition of ATP, declined to one-third when Na-free seawater replaced the normal seawater outside the axon, and then reached a stable base line. This experiment

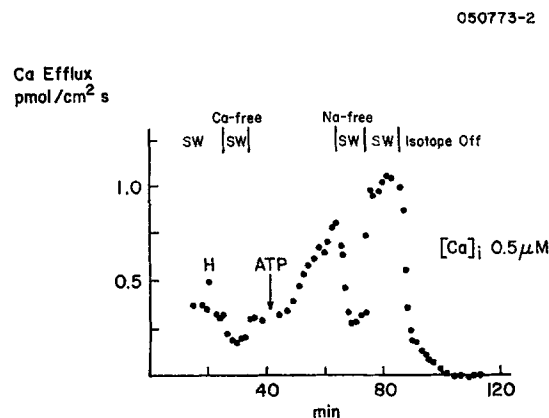


FIGURE 9. Ca efflux is shown with time for an axon dialyzed with a substrate-free solution for 1 h before efflux measurements were begun. The axon responded to a 10-mV hyperpolarizing pulse and to Ca-free seawater. When 5 mM ATP + 5 mM ArgP were added to the dialysis fluid, Ca efflux rose, and was sensitive to Na-free conditions.



confirms the report of DiPolo (1973; see *Note Added in Proof*) that ATP enhances Ca efflux.

#### DISCUSSION

##### *Efflux of Calcium in Chelated Form*

It seems likely that all experimental investigations of Ca fluxes in nerve fibers will require the use of Ca buffers to control the level of ionized Ca rather than relying on the physiological controls provided by structures such as the mitochondria or membrane pumps. Ca buffers ought to be present at a concentration of the same order of magnitude as that of the total Ca in the fiber (0.5 mM) since a priori one cannot be certain that most of the Ca in the fiber will remain fixed or complexed during the experimental procedures employed. Given these considerations, the leakage of Ca chelated to various substances from the fiber may represent an important contribution to the measured  $^{45}\text{Ca}$  loss from the nerve; stated another way, a large correction of values for Ca efflux may have to be made when the leak of Ca buffer is allowed for.

Our measurements show that when  $[\text{Ca}]_i$  is greater than 1.0  $\mu\text{M}$ , such leak corrections are quite negligible (of the order of 2–5%) but at low  $[\text{Ca}]_i$  they may be of the order of 50–100% of the measured Ca efflux. If  $[\text{Ca}]_i$  is of the order of  $10^{-6}$  M or less, then it is clear that the leak of Ca buffer at an internal concentration of 1 mM is appreciable.

##### *Effect of Potential on Calcium Efflux*

Turning to the effect of membrane potential on Ca efflux, the following statements summarize the results obtained. (a) In the absence of both  $\text{Ca}_o$  and  $\text{Na}_o$ , Ca efflux is independent of membrane potential. (b) The fractional increase in Na + Ca-dependent Ca efflux with an increase in membrane potential is greatest at low  $[\text{Ca}]_i$  and declines at the highest values of  $[\text{Ca}]_i$  studied.

Statement *a* is understandable on the basis that unequal quantities of electric charge are involved in the exchange of Na and Ca across the membrane. If there is no  $\text{Na}_o$ , then there can be no potential sensitivity of the Ca efflux. Statement *b* is understandable most easily if the stoichiometry of Na/Ca exchange is not fixed at a particular value, but is free to vary depending upon the work to be performed in Ca transport. The highest values we have observed are somewhat greater than an  $e$ -fold increase in Ca efflux per 25-mV increase in membrane potential (corresponding to the net movement of a single charge per  $\text{Ca}^{++}$  transported, or a coupling ratio of 3 Na/Ca). Such values, however, were only seen at very low  $[\text{Ca}]_i$  (0.05  $\mu\text{M}$ ); at high

$Ca_i$  ( $230 \mu M$ ), there was only one-third to one-fourth as much increment in Ca efflux. An obvious explanation of this finding is that as  $[Ca]_i$  increases, an increasing fraction of the Ca efflux is Ca/Ca exchange, and that if this were allowed for, the sensitivity of Ca efflux to membrane potential would remain constant. Another possibility is that the carrier really is more electrogenic at lower  $[Ca]_i$ . If the predominant mechanism for providing energy for calcium extrusion is coupling to sodium entry, then as the internal calcium is lowered, more sodium ions must enter per calcium extruded, regardless of the absolute level of calcium efflux. This would make the Ca/Na exchange inherently more electrogenic, in accordance with the present findings.

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