

Calcium Content and Net Fluxes in Squid Giant Axons

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ABSTRACT Axons freshly dissected from living specimens of the tropical squid *Dorytheutis plei* have a calcium content of 68 $\mu\text{mol/kg}$ of axoplasm. Fibers stimulated at 100 impulses/s in 100 mM Ca seawater increase their Ca content by 150 $\mu\text{mol/kg}\cdot\text{min}$; axons placed in 3 Ca (choline) seawater increase their Ca content by 12 $\mu\text{mol/kg}\cdot\text{min}$. Axons loaded with 0.2–1.5 mmol Ca/kg of axoplasm extruded Ca with a half time of 15–30 min when allowed to recover in 3 Na seawater. The half time for recovery of loaded axons poisoned with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and iodoacetic acid (IAA) is about the same as control axons. Axons placed in 40 mM Na choline seawater (to reduce chemical gradient for Na) or in 40 mM Na, 410 mM K seawater to reduce the electrochemical gradient for Na to near zero either fail to lose previously loaded Ca or gain further Ca.

INTRODUCTION

Although there have been extensive studies in injected squid axons of the manner by which Ca efflux varies as the ionic composition of the external medium changes (Blaustein and Hodgkin, 1969; Baker and McNaughton, 1976), corresponding changes in Ca influx are relatively less well worked out because of the greater technical difficulty in making such measurements (Baker et al., 1969). Studies of how Ca fluxes are modified by changes in both internal and external ions which can be easily accomplished by dialysis (Brinley et al., 1975; Blaustein and Russell, 1975; DiPolo, 1973; Requena, 1978) have shown that Ca efflux is a complex function of Na_o , Na_i , Ca_o , and Ca_i .

Changes in $[\text{Ca}]_i$ (the ionized Ca concentration) produced by altering $[\text{Na}]_o$ or by stimulation have been followed using either aequorin or arsenazo III (Baker et al., 1971; DiPolo et al., 1976; Requena et al., 1977; Brinley et al., 1977 *a*), but these measurements may not necessarily indicate the net Ca flux across the axon membrane.

Recently, Brinley et al. (1977 *b*), have shown that $[\text{Ca}]_T$, the Ca content of a squid axon, and $[\text{Ca}]_i$ appear to be related such that $[\text{Ca}]_i$ is about 1/1,000th of $[\text{Ca}]_T$. In order to follow up this sort of measurement, the $[\text{Ca}]_T$ of intact squid axons has been examined under conditions where the axon was either stimulated in 100 mM Ca seawater or was held in Na-free seawater to increase the $[\text{Ca}]_T$ of the fiber. Subsequent analysis of such Ca-loaded fibers enables one to estimate the net flux of Ca necessary to produce the observed analytical value.

A return of such Ca-loaded axons to seawater of various ion compositions enables one to measure the net Ca flux produced as the $[Ca]_T$ of the fiber falls.

The use of the inhibitor combination (1 mM IAA + 10 μ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, FCCP) reduces ATP_i to low levels and the loading and unloading of Ca from such poisoned fibers has also been studied. The results show that freshly dissected axons isolated from living squid have a $[Ca]_T$ of 68 μ mol/kg of axoplasm but loading either by stimulation or by treatment with Na-free (choline) seawater can increase $[Ca]_T$ to 500–3,000 μ mol Ca/kg of axoplasm. Upon transfer of a Ca-loaded axon to 3 Ca (Na) seawater, the $[Ca]_T$ of the axon falls with a half time of \sim 30 min; this recovery is independent of whether the axon has a normal ATP or an ATP content that has been greatly reduced by inhibitors. Recovery from a Ca load does not take place if the axon is placed in solutions where Na_o is only 40 mM, or if the seawater for recovery has an elevated K concentration.

METHODS

Material

Squid used in this investigation were live specimens of a tropical variety, *Dorytheutis plei*, obtained from the collecting station of Instituto Venezolano de Investigaciones Cientificas at Mochima, Venezuela and transported to the laboratory in Caracas for use.

Solutions

External solutions used in these experiments are listed in Table I. The osmolality of all solutions used was determined by comparison of the dewpoint of standard and unknown solutions using a commercial psychrometer (Wescor, Inc., Logan, Utah); they were adjusted to 1,010 mosmol/kg as measured against NaCl standards, and were SO₄²⁻-free to avoid complexing Ca.

ATP Analysis

The ATP content of axoplasm samples was analyzed using the firefly flash method described by Mullins and Brinley, 1967. Axoplasm was weighed and ground with sand in 0.1 N perchloric acid, the acid was neutralized with Tris buffer, and then the sample was assayed for ATP.

Collection of Axoplasm for Ca Analysis

The hindmost giant axons from the stellar ganglion isolated from freshly killed specimens were used. The fibers were quickly dissected from the mantles in natural seawater and lightly cleaned (a procedure which required \sim 15–30 min). Samples for analyses were obtained by extruding axoplasm from these axons. The axons were washed for 5 min in a choline, 0 calcium seawater or iso-osmolar sucrose, then blotted for a few seconds on dry filter paper, and cut at one end. The cut end was then laid on a piece of parafilm carefully cleaned with EGTA solution (250 mM) and the axoplasm extruded from the cut end by gently pressing on the fiber with a finger wrapped in parafilm. Great care was taken that no fluid along the outside of the axon was forced onto the sample, and that axoplasm near either end of the axon was not taken for analysis. Immediately after extrusion, the samples of axoplasm were aspirated into polyethylene tubes (PE 100, Clay-Adams, Div. of Becton, Dickinson & Co., Parsippany, N.J.). The mass of the axoplasm was obtained either by reweighing the tube or by measuring the

length of the sample in the tube and calculating the volume of axoplasm. The tubes were then sealed at either end with the aid of heat from a flame and stored at -20°C until analysis.

Analytical Methods

The samples were analyzed in a graphite furnace attachment (model HG 2100) to a model 305B Perkin-Elmer atomic absorption spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) using a Zn, Mg, Ca multi-element hollow cathode lamp. A ramp attachment was used to permit gradual increments in the temperature during charring and atomization. The following instrument parameters were found convenient. Dry cycle: 70 s ramp ambient to 150° with 20 s additional drying at 150°C . Charring temperature: 10 s ramp from 150° to $1,000^{\circ}\text{C}$ with 20 s additional charring at $1,000^{\circ}\text{C}$. Atomization: 6 s ramp from 1,000 to $2,700^{\circ}\text{C}$ with 2 s additional atomization at $2,700^{\circ}\text{C}$. A reducing atmosphere inside the graphite tube was maintained by passage of 5 ml/s of purified nitrogen through the center of the graphite tube. A deuterium arc was used to provide a continuous spectrum to compensate for broad band absorption from the sample matrix. With these parameters the overall sensitivity of the instrument at 423

TABLE I
COMPOSITION OF SOLUTIONS USED

Solution	Na	K	Mg	Ca	Choline	Li	Cl
	<i>mmol/liter</i>						
Seawater	440	10	50	10	—	—	580
3 Ca seawater	440	10	50	3	—	—	566
100 Ca (Na) seawater	225	10	50	100	130	—	675
3 Ca (choline) seawater	0	10	50	3	440	—	566
3 Ca (40 Na) choline seawater	40	10	50	3	400	—	566
High-K seawater	40	410	50	3	—	—	566
100 Ca (Li) seawater	0	10	50	100	130	225	675

All solutions contained 0.1 mM Na_2EGTA and 10 mM Tris Cl pH 7.6. Osmotic pressure was adjusted to 1,010 mosmol/kg.

nm was 0.004 absorbance units per picomole of calcium. This value is approximately three times less than the maximum specified sensitivity for the instrument using a nitrogen-reducing atmosphere, different ramp parameters, and less rapid nitrogen flow. This reduced sensitivity was advantageous because otherwise the maximum amount of calcium producing an absorbance change in the linear range of the instrument (i.e., 0–0.5 absorbance unit) was inconveniently small.

Atomic absorption spectroscopy using sample vaporization in a graphite tube is subject to substantial interference by the sample matrix. In the present experiments two types of interference were identified.

(a) Broad band absorption by the matrix, presumably due to organic material being atomized with the calcium. This interference was compensated for by use of a deuterium background corrector. The adequacy of the deuterium arc for broad band absorption correction was evaluated by analyzing aliquots of a solution of potassium isethionate and potassium phosphate, simulating the concentration of these materials expected in the aliquots analyzed. The samples were analyzed at a Cr line (425.4 nm), on the assumption that any contaminant Ca in these samples would not be detected by the phototube at that wavelength, but any nonspecific broad band absorption generated by atomization would be. The deuterium arc background corrector was then adjusted until no absorption could be observed during atomization at the Cr line.

(b) Specific interference on the calcium signal 423.6 nm from material in the matrix possibly due to the approximately 15-fold excess of phosphate over calcium in axoplasm. This interference was corrected by the method of standard addition, described below.

Preparation of Samples for Analyses

100 or 200 λ of 1 N nitric acid was pipetted into a platinum boat. Aliquots of the nitric acid in the boat were then tested for calcium content. The amount of calcium in the nitric acid was usually between 5 and 10% of that in the sample, on a few occasions as much as 25%.

The sealed polyethylene tube containing axoplasm was dipped in a beaker of freshly poured double-distilled water and agitated for a few seconds with a pair of clean forceps. One end of the sealed polyethylene tube was then cut and a length of PE 20 tubing, connected to a 1-cm³ syringe was forced into the inside of the PE 100 tubing. The other end of the sealed plastic tube was then rewashed in double-distilled deionized water and cut at the end, and the tip of the plastic tubing was immersed in the nitric acid. Gentle pressure on the syringe forced the sample of axoplasm into the nitric acid boat. Usually transfer was quantitative. If necessary the inside of the plastic tube was rinsed by gentle suction of nitric acid into the interior of the tube and discharged into the boat. The boat was then covered with a small glass beaker containing moistened filter paper to provide a water-saturated atmosphere, and the sample allowed to stand for \sim 5 min. The axoplasm was then broken up by tearing and teasing it with two well-cleaned Eppendorf pipette tips (Brinkman Instruments, Inc., Westbury, N.Y.). Usually the axoplasm sample could be completely dispersed this way; occasionally lumps of axoplasm remained.

In order to demonstrate that this method of preparing the axoplasm led to a quantitative extraction of calcium, in several of the early analyses, the axoplasm was deliberately left in large clumps. After the initial aliquot of nitric acid had been used for analysis, the lumps of axoplasm were reextracted with an additional 100 μ l of nitric acid, this time the axoplasm being forcefully broken up by vigorous tearing with the Eppendorf tips. The second aliquot of nitric acid was then analyzed in the same way as the first. In no case was the second extraction more than 5% of the first, and it is probable that some of the apparent calcium in the second extraction was actually a contamination from the Eppendorf pipettes, because in other experiments it was demonstrated that vigorous rubbing of pipette on the floor of the platinum boat led to increased calcium contamination of the nitric acid in the boat. It is concluded that all of the calcium contained in the axoplasm was liberated into the nitric acid solution during the first extraction.

5–10 μ l of HNO₃ were introduced into the furnace and analyzed. In addition to plain aliquots of nitric acid, aliquots were also analyzed by the method of standard additions, i.e., to some aliquots of nitric acid solution, known amounts of standard calcium chloride solution were added. Because the sensitivity proved to be dependent on the volume of fluid introduced into the furnace, care was taken so that the volume was always the same regardless of whether sample alone or sample plus standard was introduced. In the cases where no standard was added, a comparable volume of distilled water was added to the sample.

The results of a typical analysis obtained using the method of standard additions is shown in Fig. 1. The solid line represents the absorbance changes produced by standard alone, standard plus 50 pmol calcium, or standard plus 89 pmol of calcium. The absorbance change can be seen to be linear with added calcium, and extrapolates to a calcium in the aliquot of 14 pmol. The importance of calibrating the instrument by the method of standard additions can be seen by comparing a calibration obtained by adding calcium in the presence of sample (solid line) with calibration obtained using only calcium

chloride (dashed line). The sensitivity of the instrument to calcium in the presence of sample was roughly 80% of that observed in the presence of calcium chloride alone.

RESULTS

ATP Content of Control and Inhibitor-Treated Axons

Although cyanide can reduce the ATP content of isolated axons to around 100 μM , the effect may take several hours to develop (Caldwell et al., 1960). To produce a more rapid decline in $[\text{ATP}]_i$ other inhibitors were used. Fig. 2 shows that incubation of axons in seawater containing 10 μM of the mitochondrial uncoupler FCCP reduces ATP_i from 1.5 mM to ~ 0.2 mM within 15–30 min. Although iodoacetic acid (IAA) in a concentration of 2 mM reduces ATP to only ~ 0.9 mM in 1 h, the combination of 2 mM IAA and 10 μM FCCP reduced the ATP_i to ~ 30 μM after 1 h of incubation.

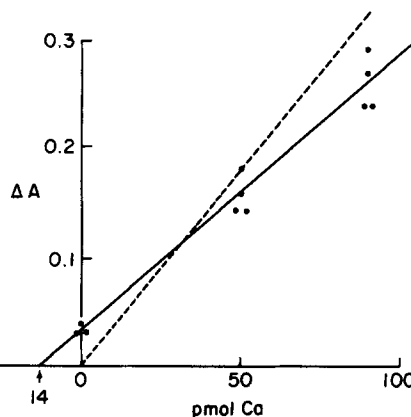


FIGURE 1. Results of standard addition of Ca are extrapolated to find the Ca content of a sample. Ordinate is the change in absorbance produced by additions of Ca to the analytical sample; abscissa is Ca added. The solid line is back-extrapolated to Ca content of sample alone. Dashed line is the uncorrected absorbance change.

The Ca Content of Fresh Axoplasm

Two previous studies of the Ca content of axoplasm (Keynes and Lewis, 1951; Blaustein and Hodgkin, 1969) showed values of 400 μmol of Ca/kg. Most of the axons used in these studies were isolated from mantles stored in chilled seawater for some hours. Inasmuch as DiPolo et al. (1976) have shown that the ionized Ca of squid axons rises with time in 10 mM Ca seawater but is stable if the seawater contains 3 mM Ca, axons used in the present study were isolated from living squid as quickly as possible and the axoplasm was promptly extruded. Preliminary studies of the $[\text{Ca}]_T$ of squid axons (Requena et al., 1977) showed a value of 70 μmol of Ca/kg (50 μM if allowance is made for dissection time in 10 Ca seawater) of axoplasm or one-eighth that of axons stored in seawater. These studies showed further that the Ca content of axoplasm was readily increased to the levels reported earlier if axons were stored for several hours in 10 mM Ca

seawater, whereas the Ca content of axons stored in 3 mM Ca seawater increased much less.

In Table II, column A, we have collected values for the Ca content of freshly isolated axons from living squid (standard axons). Inasmuch as it takes 10–15 min to isolate one axon from the mantle during which time the axon is exposed to 10 mM Ca seawater in the dissection chamber, a correction to the analytical values is necessary to compensate for the net flux of Ca that is involved. In that Requena et al. (1977) have found net Ca flux into fibers immersed in 10 Ca seawater to be 60 $\mu\text{mol}/\text{kg}\cdot\text{h}$, the axons listed in column A should have gained

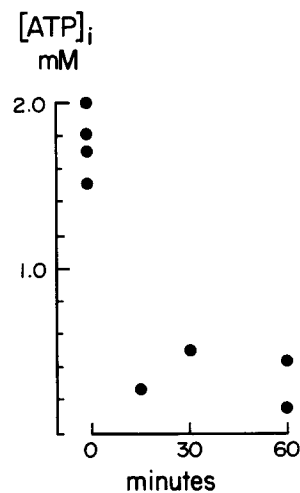


FIGURE 2. Analysis of several axons for ATP at zero time, and the ATP content of other axons treated with 10 μM FCCP in seawater for the times indicated.

about 15 $\mu\text{mol}/\text{kg}$ during the isolation procedure. Therefore, Ca content of an *in situ* axon should be about 53 $\mu\text{mol}/\text{kg}$ of axoplasm, or about 20% lower than the value of a standard (or freshly dissected) axon.

Increasing the Ca Content of Axoplasm

Baker et al. (1969) showed that the application of Na-free Li or choline seawater to squid axons greatly increased Ca influx, and Blaustein and Hodgkin (1969) have shown that Na-free solutions decreased Ca efflux, hence the expected effect of Na-free seawater is to increase the $[\text{Ca}]_T$ of an axon. Baker et al. (1971) and DiPolo et al. (1976) showed that ionized $[\text{Ca}]$ increased in axons exposed to Na-free (Li) or (choline) seawater, and Requena et al. (1977) found substantial increase in Ca content in axons exposed to 3 Ca choline seawater. These measurements have been extended in this study to include the effect of inhibitors on net entry. The results are in Fig. 3, which shows two series of experiments in which axons were soaked in either 1 mM Ca or 3 mM Ca (choline) seawater in the presence or absence of FCCP and or IAA. There was no clear effect of inhibitors on Ca gain and the mean increase was 0.25 and 0.5 mmol/kg of axoplasm per hour for axons exposed to 1 or 3 mM Ca choline seawater, respectively.

TABLE II
Ca CONTENT OF SQUID AXONS (*Dorytheutis plei*, 18°C)

A Standard axons		B Axons in 3 Ca choline seawater 1 h		C Axons stimulated in 100 Ca (Li or Na) seawater (100 impulses/s for 10 min)	
Axon ref.	[Ca] mmol/kg	Axon ref.	[Ca] mmol/kg	Axon ref.	[Ca] mmol/kg
SC 25c	0.12	S 23*	1.03	S 1*	1.85
-O 8	0.083	S 24‡	0.53	S 2‡	2.23
-O 9	0.107			S 2*	1.30
O 51	0.038	F 40*	0.52	S 3‡	1.38
O 52	0.069	F 40‡	0.54	S 3*	1.86
O 53	0.071	F 41*	0.91	S 4*	0.95
O 54	0.075			S 5‡	1.82
A 16	0.028	Mean	0.71±0.11	S 5*	1.40
B 16	0.020			S 6‡	1.10
Mean	0.068±0.023			S 6*	1.70
				S 30‡ (Li)	1.04
				S 30* (Li)	2.35
				S 31* (Li)	0.96
				Mean	1.53
				Mean‡	1.63±0.94
				Mean*	1.51±0.68

* Axons treated for 1 h with 1 mM IAA + 10 μM FCCP in 3 Ca (Na) seawater. Other axons were untreated.
‡ Control.

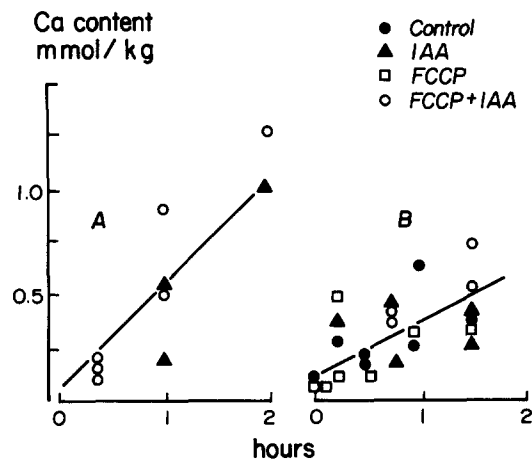


FIGURE 3. The analytical Ca content of axons is plotted as a function of time for two series of axons treated with the various inhibitors as indicated. (A) Axons were in 3 Ca (choline) seawater; (B) axons were in 1 Ca (choline) seawater.

The results of these two series of experiments and several others are collected in Fig. 4 which shows the rate of Ca entry plotted against $[Ca]_o$ for axons incubated 1–2 h in choline seawater. The data show that Ca gain from choline seawater increases less than proportionally with Ca concentration or not at all. The results suggest that in choline seawater the principal pathway for Ca gain is via the Na:Ca exchange mechanism which is near saturation over the range 1–10 mM Ca_o , in contrast to the influx in Na seawater which has been shown to be linear over a wide range of external calcium concentrations (Hodgkin and Keynes, 1957; Baker and McNaughton, 1976).

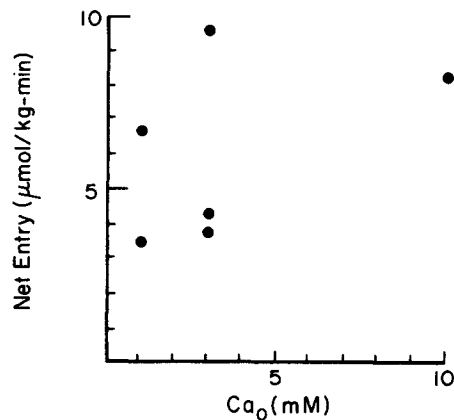


FIGURE 4. The analytical gain of $[Ca]_T$ (in $\mu\text{mol}/\text{kg}\cdot\text{min}$) is plotted against time for axons in (choline) seawater for 1–2 h as a function of the $[Ca]$ of the seawater. The point at 10 mM Ca_o is from Requena et al. (1977).

Another method of increasing the influx of Ca is by stimulation in Ca seawater. Hodgkin and Keynes (1957), and Brinley et al. (1977 *a*), showed that stimulation at 100 impulses/s in 112 mM Ca increased influx of the order of 11.5 $\text{pmol}/\text{cm}^2\cdot\text{s}$ with very little increase in efflux. These unidirectional fluxes predict a significant net gain of calcium during stimulation. Data in column C of Table II confirm this prediction by direct analytical measurement and indicate a net gain of about 0.25 $\text{pmol}/\text{cm}^2\cdot\text{s}$. As in the case of axons soaked in choline seawater, the presence of FCCP and IAA had little effect upon the net entry.

Ca Extrusion from Loaded Axons

The data in the previous section demonstrated that axons could be loaded either by soaking in sodium-free solutions or by stimulation in high Ca seawater. The results presented in this and subsequent sections examine the circumstances under which loaded axons can extrude Ca.

Fig. 5 shows the results of two series of experiments in which axons loaded with Ca by soaking in sodium-free solutions were allowed to recover for variable periods in 3 Ca seawater. In order to study the effect of ATP upon net extrusion, some axons were pretreated for 1 h with 1 mM IAA plus 10 μM FCCP. In the experiment shown in Fig. 5 A the axons initially had a $[Ca]_T$ of ~

500 $\mu\text{mol/kg}$ of axoplasm after 60 min of loading in 3 Ca choline seawater; those in Fig. 5 B had an initial content of $\sim 250 \mu\text{mol/kg}$ of axoplasm after 20 min of loading in 1 Ca choline seawater. In either case, $[\text{Ca}]_i$ is reduced to $\sim 100 \mu\text{mol/kg}$ of axoplasm with a half-time of $\sim 15\text{--}30$ min. The final content, 100 $\mu\text{mol/kg}$ of axoplasm, is larger than the content of *in situ* axons, but about that to be expected for axons immersed for 2–3 h in 3 Ca seawater, i.e., the duration of the experiment, (Requena et al., 1977). The conclusion is that all of an exogenous load can be lost by an axon bathed in seawater with a normal $[\text{Na}]$ and a $[\text{Ca}]_o = 3 \text{ mM}$.

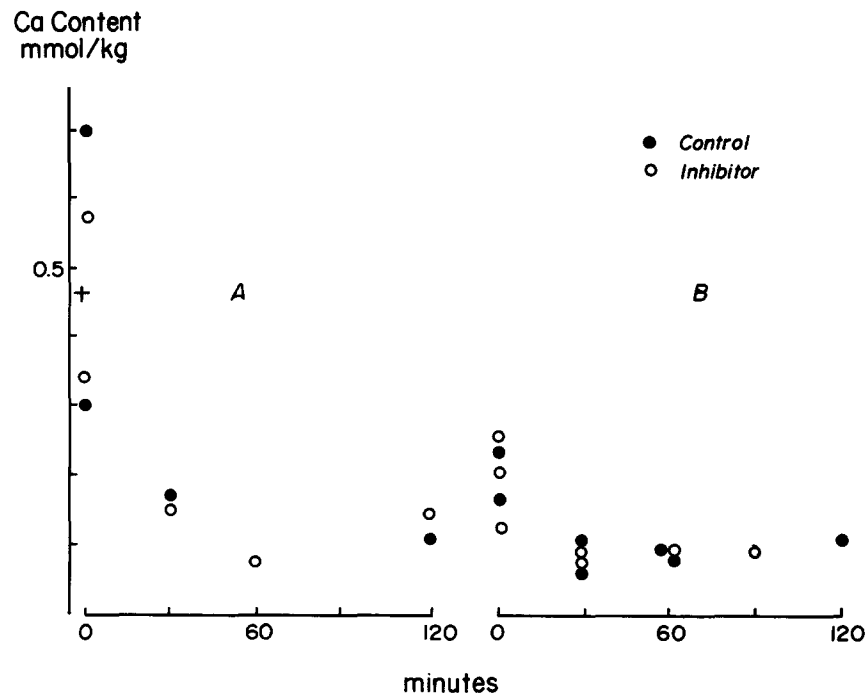


FIGURE 5. The analytical Ca content of axons loaded in choline seawater is plotted as a function of time for axons recovering in 3 Ca (Na) seawater. (A) Axons were loaded 1 h in 3 Ca (choline) seawater and then treated with either FCCP and IAA or with IAA alone in the 3 Ca (choline) loading solution for 1 h. Axons were effectively loaded for 2 h. (B) Loading was for 20 min in 1 Ca (choline) seawater \pm the inhibitor FCCP. The symbol (+) indicates a mean value.

Similar experiments were performed to determine the ability of axons to extrude Ca after they had been loaded by rapid stimulation in high Ca solutions rather than by soaking in choline seawater. The results, shown in Fig. 6, for axons loaded with $\sim 1.5 \text{ mmol Ca/kg}$ of axoplasm, by stimulation for 20 min in 100 mM Ca seawater are similar to those illustrated in Fig. 5 A and B for axons extruding Ca after loading for 1 h in choline seawater.

Considered together, the data in Figs. 5 and 6 show that axons loaded by either method, with sufficient Ca to raise the internal content by 0.2–1.5 mmol

Ca/kg of axoplasm, extrude essentially all of the exogenous load with a half-time of 15–30 min, and this extrusion is independent of the presence or absence of ATP.

The fact that the rate of extrusion of an imposed load is about the same for intact and poisoned axons is probably due to the large difference in free calcium in the two kinds of fibers (see Discussion).

The Effect of Altering the Na Electrochemical Gradient on the Ca Content of Axons

To evaluate the Na electrochemical gradient, we need information regarding $[Na]_i$ under the various experimental conditions we have employed. Both of the experimental conditions used to increase analytical Ca affected Na_i , although in

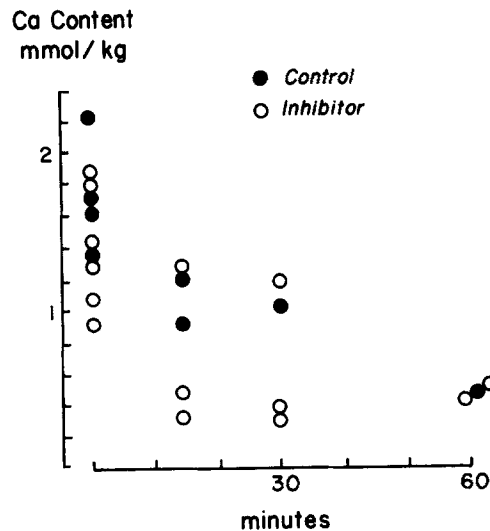


FIGURE 6. Axons loaded by stimulation in 100 mM Ca (Na) seawater for 10 min at 100 impulses/s were allowed to recover in 3 Ca (Na) seawater for the times indicated. Pretreatment of inhibited axons was with 1 mM IAA + 10 μ M FCCP for 1 h in 3 Ca (Na) seawater before stimulation. Control axons were in 3 Ca (Na) seawater for a similar time.

different ways. The $[Na]_i$ of fresh *D. plei* axons is of the order of 30 mM (Caldwell de Violich and Requena, 1977). Exposure of the nerve to 0 Ca choline seawater reduces this Na_i \sim 10 mM/h, while stimulation (60,000 impulses) in 100 Ca 225 Na seawater increased the Na content of the fiber to 70–80 mM. Fibers stimulated in 100 Ca 225 Li seawater had a $[Na]_i$ of 12 mM at the conclusion of stimulation.¹

Two series of experiments were done to determine if alteration in the sodium electrochemical gradient can affect the ability of loaded axons to extrude Ca. In the first, the chemical gradient for Na was reduced by changing the ratio of

¹ Caldwell de Violich, M., and J. Requena. Unpublished data.

external to internal Na from the normal value of ~ 10 to ~ 1 . The axons were loaded with about 1.6 mmol Ca/kg of axoplasm by stimulation in 100 mM Ca (Li) seawater. They were then allowed to recover in a solution containing 3 mM Ca, 40 mM Na, and 410 mM choline. The rationale behind these solution changes is as follows.

The initial Na content of axons subjected to Ca loading by stimulation is reduced below the normal level of 40 mM if stimulation for 20 min is carried out in Li seawater. This, in fact, reduces the Na_i to ~ 20 mM. During a recovery period in 3 Ca (Na) seawater, fibers will inevitably gain some Na. Although the $[Na]_i$ varies during the recovery period, the ratio between $[Na]_o$ and $[Na]_i$ is never greater than 2 and declines during recovery as the fibers gain Na_i .

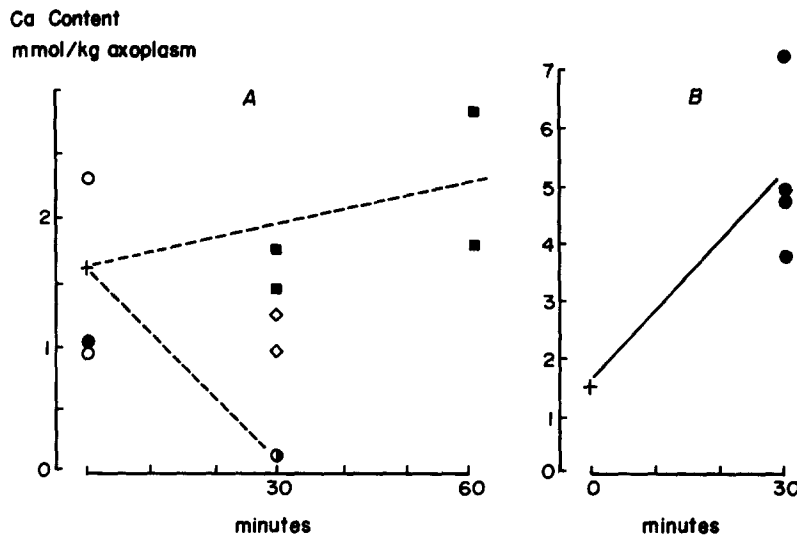


FIGURE 7. (A) Axons stimulated in 100 Ca (Li) seawater (●, control; ○, inhibitor) and allowed to recover in 3 Ca (Na) (●) or in 3 Ca, 40 Na (choline) seawater (■) or the above solution with inhibitors (◇). (B) Axons loaded as in (A) and allowed to recover in 3 Ca, 40 Na, 410 K seawater, with 100 nM TTX.

Therefore, the data in Fig. 7 show the inability of axons to extrude Ca under circumstances in which the $[Na]$ ratio is near unity. The results indicate that, in contrast to axons with a normal Na gradient (i.e. half-filled circle in Fig. 7 A), axons with a reduced Na gradient do not extrude Ca during the first 30 min of recovery and may actually gain some Ca.

In a second series of experiments, both the chemical and electrical gradients for Na were reduced to near zero by making Na_o 40 mM and depolarizing the fiber with 410 mM K_o .

Inasmuch as depolarization increases the leakage of sodium across the membrane (Brinley and Mullins, 1974), at least partly by opening voltage-dependent Na channels, 100 nM tetrodotoxin was added to the bathing medium to inhibit any Ca movement through the Na channels. Fig. 7 B shows the Ca

content of fibers first loaded by stimulation at 100 impulses/s in 100 mM Ca seawater and then bathed in 3 mM Ca, 410 mM K, 40 mM Na seawater with TTX. In this solution the Ca content rose to about 5 mmol/kg of axoplasm after 30 min of incubation. The fact that the $[Ca]_T$ exceeds extracellular $[Ca]$ (with E_m near zero) undoubtedly reflects the ability of the cytoplasmic buffering capacity to maintain a low ionized Ca so that a net inward chemical gradient for Ca exists even though the $[Ca]_T$ rises above that outside the fiber.

The conclusion from these experiments is that Ca-loaded axons require both an electrical and chemical gradient for Na if the axon is to extrude a calcium load at a measurable rate.

DISCUSSION

Energy Sources for Ca Homeostasis

The results of this study complement measurements of ^{45}Ca fluxes, and of measurements of $[Ca]_i$ by aequorin and by arsenazo in showing that a requirement for the maintenance of a normal $[Ca]_T$ in the squid axon is the presence of Na_o . The exact concentration of Na required has not been fixed by the present experiments but both isotope and aequorin studies show that below $Na_o = 180$ mM, a net flux develops and Ca_i begins to rise.

Our measurements show that analytical Ca of the fiber rises linearly with time for periods up to 2 h if $[Na]_o = 0$ (3 Ca choline) while Ca content appears to remain high and constant at around 2 mmol Ca/kg of axoplasm if $[Na]_o$ is 40 mM. If $[Na]_o$ is made 440 mM, then the Ca content of the axon rapidly declines to control levels.

If the membrane is depolarized by increasing the $[K]$ of seawater, the Ca content of the fiber increases to very high levels even though $[Na]_o$ is maintained at 40 mM. This agrees with a finding using aequorin (Requena et al., 1977) that $[Ca]_i$ is increased if the membrane is depolarized.

Finally, axons can recover from an applied Ca load whether or not inhibitors that reduce ATP to low levels are present. This agrees with findings using aequorin that axons loaded with Ca can recover to control values of $[Ca]_i$ even though apyrase has reduced ATP (Requena et al., 1977). Although inhibitors vary in the extent to which they can reduce $[ATP]_i$ in an intact axon, both apyrase and FCCP-IAA can reduce ATP_i to levels of 20–30 μM . Inasmuch as DiPolo (1974) shows that ATP affects Ca efflux with a K_m of ~ 250 μM , this reduction should be adequate to highly inhibit ATP-driven Ca pumping.

To summarize the foregoing findings, the extrusion of Ca from the axon clearly requires the performance of work, and in the virtual absence of ATP (< 30 μM) the only clear source of free energy is the Na electrochemical gradient.

If the Na electrochemical gradient is the energy source for Ca movement, then the direction of the net Ca flux can be expected to be inward if

$$r(E_{Na} - E) - z(E_{Ca} - E) \quad (1)$$

is negative (where r is the coupling ratio Na/Ca and z is the ion valence), whereas the axon must lose Ca if this expression is positive.

Information regarding the Na electrochemical gradient has been given earlier and is only summarized here:

Treatment	[Na] _i
Fresh axon	30 mM
0 Ca choline seawater	-10 mM/h
Stimulation 6×10 ⁴ pulses in 100 Ca seawater	75 mM
Stimulation 6×10 ⁴ pulses in 100 Ca (Li) seawater	12 mM

The metabolic inhibitors used to reduce ATP also affect Na_i. Treatment of axons bathed in 3 Ca ASW plus FCCP and IAA gain Na at a rate of about 30 mM/h, whereas the Na content of fibers kept in 3 Ca choline seawater is virtually unchanged.¹

Although stimulation increases internal sodium this does not seem to affect Ca loading because the Ca content of fibers stimulated in either Na or Li seawater was about the same. This result is probably due to the fact that Ca enters through the Na channel during stimulation, hence such Ca loading cannot be expected to depend upon Na_i except indirectly as the generation of an action potential depends upon the Na gradient across the membrane. This gradient of course decreases as stimulation proceeds.

The above information on [Na]_i can be used to analyze the data shown in Fig. 5 A with respect to the direction and magnitude of the difference in electrochemical gradients as shown in Table III. Here, initial Ca loading was taken as 0.5 mmol/kg and ionized [Ca] as 1/1000th of this, while [Na]_i was taken as that given above for a 1-h soak of the axon in Na-free seawater, [Na]_o given the nominal value of 1 mM, r assumed to be 4, and the membrane potential E as -60 mv throughout. Clearly the analysis shows that under loading conditions there is a large gradient difference favoring Ca entry, whereupon changing the external seawater to 3 Ca (Na), the sign of the gradient reverses as does the direction of the net flux. When fluxes are in balance at the end of the unloading period (zero net flux) there is still a gradient difference favoring Ca extrusion. This is to be expected because some Ca entry takes place via leakage pathways and to balance this the Na/Ca carrier must produce a net outward flux in the steady state, or Eq. 1 must have a positive value.

The existence of a Ca leak makes it necessary to view the analysis just made with some caution, because the magnitude of the net flux is a kinetic rather than a thermodynamic variable. If the magnitude of the carrier-mediated Na/Ca exchange is small compared with the leak, the operation of the carrier will have no effect on the net flux. An example of this is shown in Fig. 7 A where [Na]_o is 40 mM, and since the fiber was stimulated in Li seawater, [Na]_i was initially 12 mM or ($E_{Na} - E$) is 90 mV and E_{Ca} is 96 mV. This works out to a gradient difference of (360 - 312) or +48 mV, yet the fiber is not losing Ca. The reason for this situation is that, apart from any inaccuracy in estimating Na_i, the magnitude of the carrier net flux is small because of the low [Na] involved and leakage tends to dominate.

TABLE III
DIFFERENCE IN Na AND Ca ELECTROCHEMICAL

Condition	[Na] _o	[Na] _i	E _{Na}	[Ca] _i
	mM	mM	mV	mM
End of 1 h in 3 Ca choline	1	20	-75	0.5
Start in 3 Ca (Na)	440	20	+78	0.5
End of unload	440	40	+60	0.05

* $4F(E_{Na} - E) - 2F(E_{Ca} - E)$ (see Eq. 1).

Equilibrium between Free and Bound Calcium

Prior work has demonstrated that >99.9% of the Ca which enters axons during loading is bound either by mitochondria or by an FCCP- and cyanide-insensitive system: this is true for loads of Ca up to 3 mM and buffers show no signs of saturation at this level (Brinley et al., 1977 *b*). The present data indicate that this bound calcium remains in equilibrium with [Ca]_i which in turn governs extrusion by the membrane pump(s). This conclusion follows from the fact that the Ca content after recovery from an imposed load is equal to that of control axons kept for the same period in 3 Ca seawater. There is no evidence that, even at very high loads, a significant fraction of Ca is retained permanently by the fiber.

Isotope Studies

Data that supports the idea that net Ca extrusion can take place was also obtained by Blaustein and Hodgkin (1969) who showed that ⁴⁵Ca efflux in injected axons was increased 10-fold when cyanide was allowed to act on axons for times long enough for ATP to fall to low levels. By contrast, Ca influx was not affected so that, as the authors pointed out, the axon must have been losing Ca against its electrochemical gradient. Similar effects using dialyzed axons have been reported by Blaustein (1977).

Rates of Calcium Extrusion in Control and Poisoned Fibers

An explanation for the fact that the extrusion of Ca from loaded fibers appears not to depend upon ATP whereas unidirectional Ca fluxes are known to be greatly increased by ATP may be that the [Ca]_i is much higher in poisoned fibers. Previous work has shown that, over a wide range of Ca loads only 0.06% of an imposed load appears as free Ca in intact fibers, whereas about 10% appears as free Ca in FCCP-poisoned fibers (Brinley et al., 1977 *b*). The [Ca]_i of poisoned fibers therefore is about 150 times greater than in unpoisoned fibers regardless of load. Since Ca efflux, as measured with tracers, increases with both internal ATP and Ca_i, one might expect that, at any given Ca load, the effect of low ATP (which would reduce Ca efflux) would be compensated by the

GRADIENTS* DURING Ca LOADING AND UNLOADING

[Ca] _i	[Ca] _o	E _{Ca}	4 (E _{Na} - E)	2 (E _{Ca} - E)	Gradient difference	Direction of Ca net flux
μM	mM	mV	mV	mV	mV	
0.5	3	+110	-60	+340	-400	inward
0.5	3	+110	+552	+340	+212	outward
0.05	3	+139	+480	+400	+80	balanced

presence of a much higher [Ca]_i in the poisoned fiber. There are, however, quantitative difficulties with this explanation.

Table IV summarizes information on control and poisoned axons shown in Figs. 5 and 6, listing total content, estimated free calcium concentration, together with estimates of Ca efflux as measured in this species of squid (Table II in DiPolo, 1973). The rates of efflux calculated from analytical data for

TABLE IV
CALCIUM CONTENT AND FLUXES IN SQUID AXONS

Text figure	Initial analytical Ca	Calculated [Ca] _i : control (C); poisoned (P)	Observed Net Ca Flux	Calculated Ca efflux	
				No ATP	2 mM ATP
	mmol/kg	μM		pmol/cm ² ·s	
5 A	0.2	0.1 C	0.5	-	0.1
		20 P		0.8	-
5 B	0.5	0.3 C	2.0	-	0.3
		50 P		1.0	-
6	1.5	1 C	8.0	-	1.0
		150 P		3.5	-

poisoned fibers with no ATP and high free calcium are reasonably concordant with the tracer measurements of DiPolo, considering the errors inherent in both measurements. However, the tracer measurements indicate that the extrusion of calcium from unpoisoned fibers should have been substantially slower, by at least a factor of three, than the poisoned fibers. The discrepancy may reflect an underestimate of the [Ca]_i after loading in this species of squid, since the value of 0.6 nM free Ca/μM Ca load for control fibers was actually obtained on *Loligo pealei*.

This work was supported by grants BNS 76-19728 and PCM 76-17364 from the National Science Foundation, NS-13402-02 from the National Institutes of Health, and 31.26.S1-0602 from the Consejo Venezolano de Investigaciones Científicas y Tecnológicas (CONICIT).

Received for publication 31 July 1978.

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