The Control of Ionized Calcium in Squid Axons

J. REQUENA, R. DiPOLO, F. J. BRINLEY, JR., and L. J. MULLINS

From the Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela, and the Departments of Physiology and Biophysics, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT Measurements of the Ca content, $[Ca]_T$, of freshly isolated squid axons show a value of 60 μmol/kg axoplasm. Axons in 3 mM Ca (Na) seawater show little change in Ca content over 4 h, while axons in 10 mM Ca (Na) seawater show gains of 18 μmol Ca/kg·h. In 10 Ca (choline) seawater the gain is 2,400 μmol/kg·h. Using aequorin confined to a dialysis capillary in the center of an axon, one finds that $[Ca]_i$ is in a steady state with 3 Ca (Na) seawater, and that both 10 Ca (Na) and 3 Ca (choline) seawater cause increases in $[Ca]_i$. In 3 Ca (Na) seawater-3 Ca (choline) seawater mixtures, 180 mM $[Na]_o$ (40% Na) is as effective as 450 mM $[Na]_o$ (100% Na) in maintaining a normal $[Ca]_i$; lower [Na] causes an increase in $[Ca]_i$. If axons are injected with the ATP-splitting enzyme apyrase, the resulting $[Ca]_i$ is not different from control axons. Such axons also recover to their initial $[Ca]_i$ after loading with high $[Ca]_o$ or low $[Na]_o$ solutions. Depolarization of an axon with 100 mM K (Na) seawater leads to an increase in the steady-state level of $[Ca]_i$ that is reversed upon returning the axon to normal seawater. Freshly isolated axons treated with either CN or FCCP to inhibit mitochondrial Ca buffering can still maintain a normal $[Ca]_i$ in 1 Ca (Na) seawater.

INTRODUCTION

Over the past 20 yr, a substantial literature has been built up on the effect of various parameters on ^{45}Ca fluxes in squid axons. This starts with a paper by Hodgkin and Keynes (1957) who established values for ^{45}Ca influx and the rate constant of efflux, and who demonstrated that both stimulation and steady depolarization with high-K seawater increased Ca influx. Further studies (Blaustein and Hodgkin, 1969) established that Ca efflux was affected by Na_o and Ca_o, that Ca influx was enhanced by low Na_o, while there was a Na efflux dependent on Ca_o, and on Na_i (Baker et al., 1969), and that Ca efflux was enhanced by ATP (DiPolo, 1973, 1974) and by increases in Ca_i (Brinley et al., 1975). More recently, it has been shown that hyperpolarization enhances Ca efflux (Mullins and Brinley, 1975) and that depolarization decreases Ca efflux (Blaustein and Russell, 1975; Mullins and Brinley, 1975; Baker and McNaughton, 1976).

This impressive array of experimental findings, while making it clear that Ca fluxes in squid axons depend on six experimental variables, viz., Na_o, Na_i, Ca_o, Ca_i, E_m, and ATP_i, does little to make it clear just how Ca homeostasis is achieved. One approach to this problem is to look not at the unidirectional

fluxes of Ca, but at the level of ionized Ca in an axon under conditions such that each of the experimental variables mentioned above is able to express its contribution to the net Ca flux across the membrane. A start on this problem (DiPolo et al., 1976) was made with a determination of the normal ionized Ca concentration in an axon under steady-state conditions of Ca flux. It was found necessary that the [Ca] of seawater be 3 mM for a steady, time-independent aequorin light emission to occur. The present studies are an extension of these earlier measurements with aequorin and they are combined with analytical measurements of the Ca content of axoplasm from fresh intact axons as measured by atomic absorption spectrophotometry to show: (*a*) that Na_o is essential for the maintenance of a normal Ca_i in axoplasm but that 180 mM Na_o is as effective as 450 mM Na_o in this respect; (*b*) that the hydrolysis of most of the ATP in the axon with the enzyme apyrase does not affect the level of ionized [Ca] in axoplasm and that raising the [Ca] of such axons with high Ca_o or low Na_o still results in a recovery to normal levels of Ca_i in the virtual absence of ATP; (*c*) that depolarization of the membrane with 100 mM K seawater leads to a marked increase in Ca_i with recovery to normal levels of Ca_i when the membrane repolarizes; and (*d*) that inhibitors such as CN and FCCP, which abolish mitochondrial buffering of Ca, still allow the axon to maintain a normal $[Ca]_i$ if $[Ca]_o$ in seawater is reduced.

Analytical studies show that the axoplasm of freshly dissected squid axons contains only 60 μmol Ca/kg axoplasm, $[Ca]_T$, but that this is readily increased by storing axons in 10 mM Ca seawater. Companion studies of changes in Ca_i under such conditions show that this rises much more slowly, or that buffering systems in axoplasm absorb 1,000 Ca^{++} for every Ca^{++} that goes to increase the ionized Ca.

MATERIALS AND METHODS

Experimental Animals

Two species of squid were used in this investigation. *Loligo pealei* (used for aequorin experiments and some analytical measurements of the Ca content of axoplasm) were obtained at the Marine Biological Laboratory, Woods Hole, Mass., during May and June 1976. *Dorytheuthis plei* (used for some analytical measurements of Ca content) were obtained from the collecting station of the Instituto Venezolano de Investigaciones Científicas at Mochima, Venezuela, and transported to the laboratory for use in January 1976. Only axons from living squid were used. Axons were dissected in seawater and then transferred to experimental solutions for subsequent use.

Aequorin

This material was a gift of Drs. O. Shimomura and F. Johnson and was prepared as previously described (DiPolo et al., 1976). Spectrophotometric measurement of the material used showed it to have a concentration as a stock solution of 200 μM; ~0.1 μl of this solution was used undiluted in the porous dialysis capillary. We are greatly indebted to Drs. Shimomura and Johnson for making this essential material available to us.

Light Emission Measurements

The same electronic and recording arrangements as have been previously described (DiPolo, 1976) were used in this study. Because it was inconvenient to move the photo-

multiplier tube in order to examine the axon from time to time, the dialysis chamber used to mount the axon was modified as shown in Fig. 1 to accommodate two light pipes with a rectangular array of glass fibers at one end, which was 2.8 × 13 mm (Dolan Jenner Inc., Melrose, Mass., type BF), while the other end was a quarter-inch circular array of glass fibers. These two light pipes were coupled to the photomultiplier. The two light pipes were brought within 2 mm of each other, with the axon in between and, by adjusting the vertical position of the light pipes relative to the axon, a maximum of the light emitted

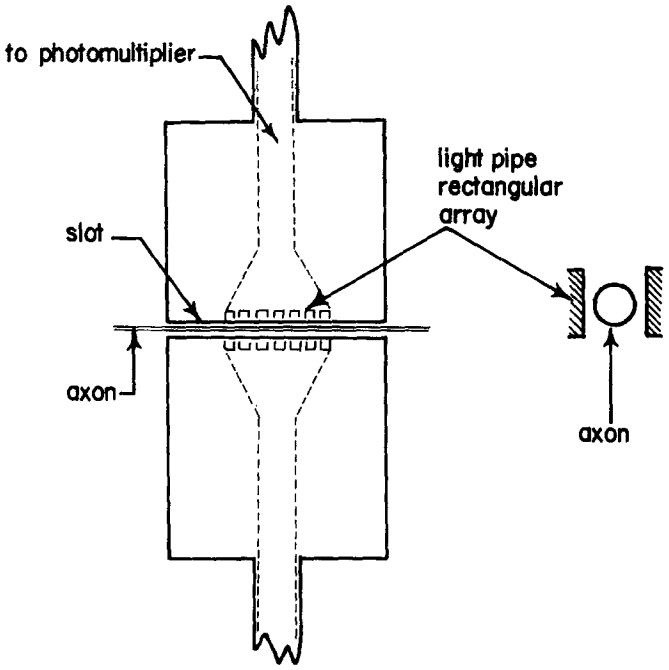

FIGURE 1. *Left*, Top view of a squid axon shown positioned between two opaque plastic blocks containing glass fibers in a rectangular array. Both light pipes are coupled to a single photomultiplier tube. *Right*, In cross-section, location of the axon relative to the fiber optics.

could be intercepted. This arrangement did not produce any increase in signal over that formerly used, presumably because although the solid angle of light intercepted was greater, losses in the light pipes and their junctions with seawater and the photomultiplier compensated for the greater light collection efficiency. The arrangement was more convenient, however, as by closing the shutter on the photomultiplier, the axon could be examined or a new drop of aequorin introduced into the porous capillary at any time. One change in the electrical system for measurement was the introduction of a 10^4 μF capacitor at the output of the amplifier. This increased the time constant for the response of the system from ~0.2 to 12 s with a substantial reduction in noise.

Apyrase Injection

Stock solutions of potato apyrase (Sigma Chemical Co., St. Louis, Mo.) 100 mg/ml of 0.5 M KTES buffer (pH 7.3) were prepared. These solutions were passed through two microcolumns of Chelex to remove Ca as a contaminant and the purified solution was collected in plastic tubes and put in storage at −70°C. Tests of 1-μl samples of this solution

in 100 μl of 10 μM ATP solution, using the firefly flash technique as an assay for ATP, showed that less than 1 μM ATP existed within 1 min after the introduction of the enzyme into the ATP solution.

For microinjection, the purified stock solution was loaded into a 100-μm capillary connected to a 1-μl Hamilton syringe and this apparatus was mounted in a horizontal microinjection assembly designed to work with a dialysis chamber. The principle was the same as the usual vertical injector but for aequorin measurements it was convenient to have the axon cannulated at both ends in a dialysis chamber, to microinject over a length of 20–25 mm, and then to introduce the dialysis capillary, center the 13-mm long porous region with respect to the apyrase injection path length, and then to introduce aequorin and begin measurements of light emission.

FCCP Solutions

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) is quite insoluble in seawater, hence solutions were made in dimethyl sulfoxide (2 mg/ml). This stock solution was taken up in a 250-μl Hamilton gas-tight syringe and the syringe was connected to the tube carrying seawater into the experimental chamber. The syringe was driven by a mechanism which delivered 1 μl/min of stock FCCP solution to the seawater flowing at a rate of 1 ml/min so that a 1:1,000 dilution was carried out continuously. This arrangement proved necessary because FCCP is readily extracted from aqueous solutions by grease used to seal the various components of the experimental chamber and by plastic tubing. The FCCP used was a generous gift of Drs. M. Blaustein and A. Scarpa.

Interpretation of Aequorin Results

The light emission from aequorin in the present experiments has been used to monitor changes in net flux of Ca across the axonal membrane or changes in the buffering capacity of axoplasm when axons were treated with inhibitors. No attempt has been made to determine precise values for ionized Ca in this study; however the light emission measurements shown in Table IV have a mean value that is virtually identical with that found in a previous study (DiPolo et al., 1976) where a calibration for Ca_i was made and found to be 20 nM. In some early experiments, whenever light emission from aequorin reached a level 15 or more times resting glow, or when an aequorin drop had been in use for 6–8 h, it was replaced with a new drop in the porous capillary. Since the light emission from such replacement drops was within 10% of the original drop's light emission, it was considered unnecessary to replace aequorin drops frequently.

The absolute value of light emission measured from aequorin depends strongly on the extent to which the axon is cleaned. Highly cleaned axons give large light emissions but are less likely to survive for long periods of time. For such reasons, cleaning the axon of connective tissue was often very lightly done. Such axons have an apparently low light emission (or low Ca_i), but since we were primarily interested in changes in light emission this procedure did not make for difficulty in the interpretation of experiments.

An isolated squid axon can be expected to undergo a continuous increase in its leakage to Ca; the rate at which this process takes place is unknown, however, and there is no entirely satisfactory procedure for measuring it. There does appear, however, to be a correlation between Ca leakage and the loss of electrical excitability of the fiber. All the fibers on which we report were electrically excitable at the end of the experiment, and all except one were capable of reducing their Ca_i to levels close to the initial resting glow. Another change taking place with time is an increase in Na_i; fresh axons can be expected to have an Na_i of about 40 mM and an initial net Na flux of about 20 pmol/cm^2 s (corresponding to a Na gain of about 6 mmol/kg axoplasm h). From Na flux data (Brinley and Mullins, 1968) there are reasons for believing that Na_i will stabilize at values of 80–90

mM. Such a change in Na_i, however, is known to affect both Ca influx (Baker et al., 1969) and Ca efflux (Brinley et al., 1975; Blaustein and Russell, 1975; DiPolo, 1976; Requena, 1976). We have no data that can be unambiguously related to this increasing Na_i with time, but the occurrence ought to be kept in mind.

We have used the inhibitors NaCN and FCCP as well as intracellular injections of the enzyme apyrase for various purposes. All these agents have in common the property of reducing ATP_i, although at quite variable rates. The results of such hydrolysis, apart from any direct effects that ATP might have on Ca fluxes, are twofold. First, there is an increase in free Mg_i which has been shown by DiPolo et al. (1976), using eriochrome blue SE and spectrophotometry, to be 1 mM, a change in Mg_i which these authors also showed to produce only a small effect on light emission by aequorin. The second effect of inhibitors or apyrase is that these treatments must be expected to increase the net flux of inward Na to ~40 pmol/cm^2 s or a net gain of 12 mmol/kg axoplasm h. In the absence of ATP, there is every reason to expect that Na_i increases with time. Since experimental conditions are bound to reflect differently in the Na_i level of normal or poisoned axon, such changes in Na_i ought to be kept in mind in interpreting the records to be presented. One of the mitochondrial inhibitors (FCCP) is a H^+ ionophore, while another (CN) is not. The effects of these inhibitors appear identical in the absence of ATP, hence the H^+ effect of FCCP does not appear to be involved.

Solutions

External solutions used in these experiments are listed in Table I. The osmolarity 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 900 mosmol/kg in *Loligo* or to 1,000 mosmol/kg in *Dorytheuthis*, as measured against NaCl standards, and were $SO_4^=$-free to avoid complexing Ca. This difference in osmolarity resulted from the use of 50 mM [Mg] with *Dorytheuthis* and 25 mM [Mg] with *Loligo* and ~5% higher [Na]. Solutions with 60–10% [Na] were made by mixing appropriate volumes of 0 Na (choline) with Na seawater.

Collection of Axoplasm for Ca Analysis

Only giant axons isolated from freshly killed specimens were used. The fibers were dissected from the mantles in flowing natural seawater (a procedure which required approximately 15–30 min), and then lightly cleaned in chilled artificial seawater containing 3 mM calcium (*Loligo*) or 10 mM Ca (*Dorytheuthis*). Samples for analyses were obtained by extruding axoplasm from these axons. Because the size and amount of surrounding

TABLE I

COMPOSITION OF SOLUTIONS USED

	External solutions					
Constituent	Na seawater	0 Ca	0 Na	0 Ca, 0 Na	3 Ca	37 Ca
			mM			
K	10	10	10	10	10	10
Na	452	462	2	2	459	417
Choline	0	0	450	462	0	0
Mg	25	25	25	25	25	25
Ca	10	0	10	0	3	37
Cl	510	500	510	500	504	549

CN seawater was made by removing 2 mmol/liter of NaCl or choline Cl and substituting 2 mmol/liter NaCN. The pH of all solutions was 7.5, and each contained 2 mM NaTES and 0.1 mM NaEGTA.

connective tissue varies considerably for the two species, slightly different methods for extrusion were used. For *D. plei*, the axons were washed for 5 min in a solution of 0 calcium, 0 sodium, 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 and the axoplasm extruded from the cut end by gently pressing on the fiber with a finger wrapped in parafilm. In the case of *L. pealei*, because of the greater amount of connective tissue surrounding the fibers, axons were washed for 10–20 min in sodium-free, calcium-free solution, then blotted, cut at the larger end, and placed on a piece of filter paper with the cut end resting in the center of a piece of clean plastic wrap. The axoplasm was then extruded from the axon by pressing on it with a Teflon roller. In both methods of extrusion, great care was taken that no fluid along the outside of the axon was forced onto the sample and that axoplasm near the end of the axon was not taken for analysis. Immediately after extrusion, the samples of axoplasm were aspirated into polyethylene tubes (PE 100). 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 by pinching with a heated forceps and stored at −50°C until analysis.

Preparation of Samples for Analysis

An axoplasm sample from a polyethylene tube was discharged into several hundred microliters of 1 N nitric acid contained in a small platinum boat. The axoplasm was allowed to remain in the acid for about 5 min and then broken up by being macerated with two well-cleaned Eppendorf pipette tips. The volume of nitric acid was adjusted so that a 10-μl sample of this volume contained approximately 50 pmol of calcium. The 10-μl sample was then introduced into the graphite furnace followed by an additional 30 μl of solution that was either distilled water or 5 μM calcium standard. The total volume for each analysis was therefore kept constant at 40 μl (constancy of total volume per analysis was important, because the sensitivity of the instrument was demonstrated to vary significantly with total volume of solution introduced).

Ca Analysis by Atomic Absorption

The samples were analyzed with a model 305B Perkin-Elmer atomic absorption spectrophotometer, by using a model HG 2100 Graphite Furnace, with ramp accessory to permit gradual increments in temperature during the drying, charring, and atomization cycles. A reducing atmosphere inside the graphite tube was maintained by passage of purified nitrogen 300 cm^3/min through the center of the tube. With these parameters the overall sensitivity of the instrument at 423 nm was 0.004 absorbance units per pmol calcium.

Broadband absorption produced by the matrix material was compensated for by use of a deuterium background corrector. The adequacy of this method of broadband absorption correction was evaluated in preliminary experiments by analyzing a solution of potassium isethionate and potassium phosphate which contained these compounds in concentrations to be expected in actual analyses of axoplasm. These samples were analyzed at 432 nm. At this wavelength the detector should be insensitive to any contaminant calcium, and should record only a nonspecific broadband absorption. The deuterium arc background corrector was then adjusted until no absorption could be observed during atomization at this noncalcium line.

RESULTS

Ca Content of Axoplasm

Early measurements of the analytical Ca content of axoplasm (Hodgkin and Keynes, 1957; Blaustein and Hodgkin, 1969) gave values of the order of 400

μmol/kg axoplasm for axons stored for some time in 10 Ca seawater before analysis. We undertook the analysis of axoplasm for Ca using atomic absorption analysis to examine the net fluxes of Ca occurring under the various experimental treatments that we have used to induce net fluxes into squid axons. Axons from squid caught in Mochima, Venezuela (*D. plei*) and in Woods Hole, Mass. (*L. pealei*) were used for this study and Fig. 2 shows the results obtained with *Dorytheuthis*. Initial values for the [Ca] of axons from living squid averaged 70 μmol/kg axoplasm, while those axons maintained in 3 Ca seawater for several

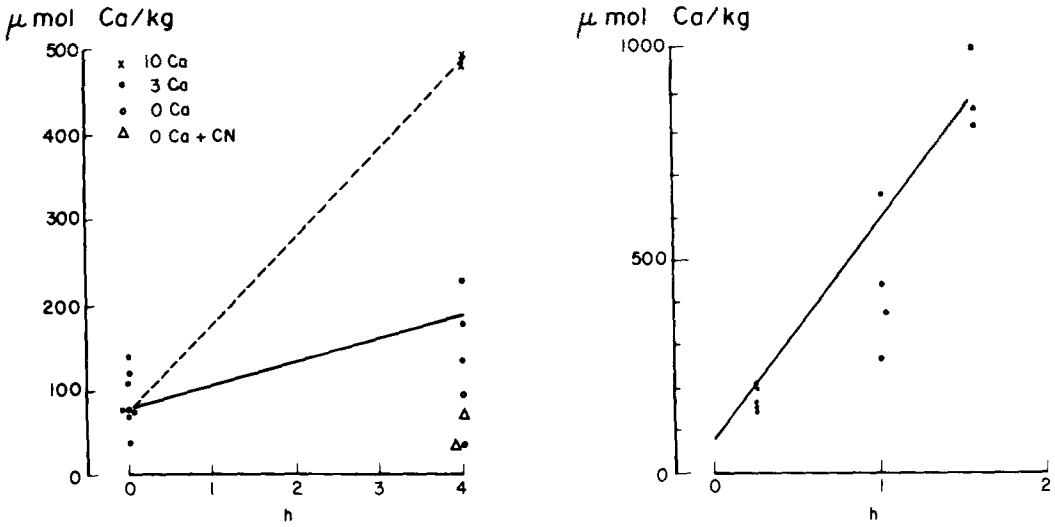

FIGURE 2. Ca content of extruded axoplasm from *Dorytheuthis* is shown as a function of time for (*left*) axons bathed in Na seawater with the [Ca] as indicated, and (*right*) axons bathed in 10 mM Ca (choline) seawater.

hours showed at most a 10% increase in this value. Axons maintained in 10 Ca seawater, by contrast showed a sevenfold increase in total Ca content in 4 h, while axons maintained in 10 Ca (choline) seawater showed a very large increase in Ca content in 1 h.

In Fig. 3 the results obtained with *Loligo* are shown. The initial value for axoplasmic Ca is somewhat lower (60 μmol/kg axoplasm), and the increase in Ca content in 10 Ca (choline) seawater is somewhat greater, but qualitatively the results are similar to those in Fig. 2 and confirm observations made with aequorin by DiPolo et al. (1976) that in 3 Ca (Na) seawater ionized Ca is in a steady state. The result is important because one could imagine that a net flux of Ca into the axon might be occurring without a corresponding change in $[Ca]_i$ as measured in the center of the axon. These analytical results show that this does not happen. An analysis of the net fluxes resulting from these experimental treatments is given in Table II.

One of the surprising results from this tabulation is the large value of the Ca net flux for *Loligo* in choline seawater; it is about twice literature values measured with isotopes, and approaches in magnitude the passive K influx into squid axons from 10 K seawater (Mullins and Brinley, 1969). The finding is

surprising in the sense that one thinks of Ca as a very poorly permeable ion, yet its flux at the same concentration (10 mM), is virtually the same as that of the most permeable ion, potassium.

Sensitivity of Aequorin to Net Ca Fluxes

If aequorin is injected into a squid axon, then as Baker et al., (1971) have shown, light emission responds quickly (within 1 min) to changes in external [Ca], but

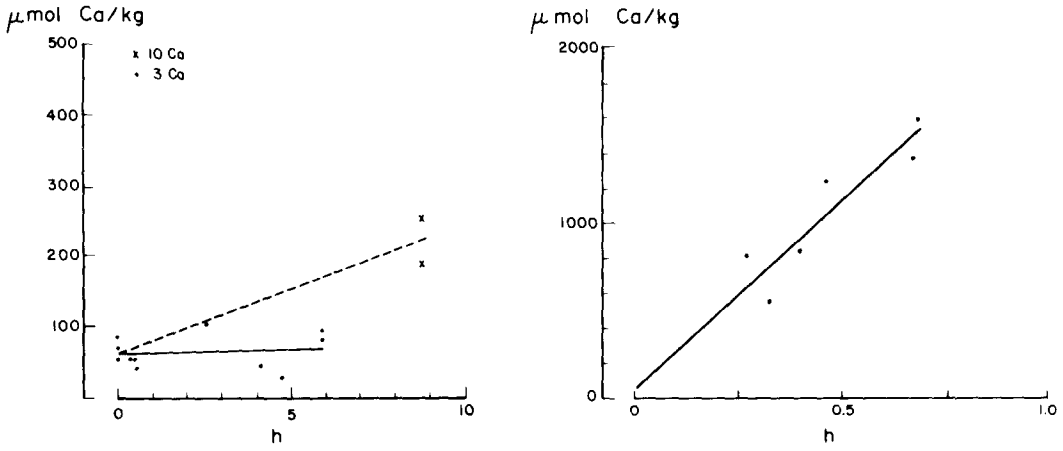

FIGURE 3. Ca content of axoplasm extruded from *Loligo* as a function of time. The axon was bathed in (*left*) Na seawater with the [Ca] shown or (*right*) 10 mM Ca (choline) seawater. T = 20°C.

TABLE II

Ca CONTENT AND NET Ca FLUXES IN SQUID AXONS

Seawater composition	Initial mean Ca content	Ca gain	Net Ca flux
	μmol/kg	*μmol/kg·h*	*fmol/cm² s*
Loligo	59 (50)*		
3 Ca (Na)		1.6	5.3
10 Ca (Na)		18.6	64.0
10 Ca (choline)		2,400.0	8,300.0
Dorytheuthis	70 (50)		
3 Ca (Na)		24.0	67.0
10 Ca (Na)		101.0	280.0
10 Ca (choline)		500.0	1,400.0

* Values in parentheses represent an extrapolation from time of analysis to zero time (death of the squid) using values for net flux in 10 Ca seawater.

this response is virtually impossible to calibrate because the shape of the Ca concentration gradient within the axon is unknown. With aequorin confined to a dialysis capillary located axially (DiPolo et al., 1976) the region of measurement of $[Ca]_i$ is as far as possible from the membrane, and the aequorin is a detector of concentration changes that result from any net flux of Ca across the membrane. The diagram in Fig. 4 attempts to illustrate the manner by which net Ca fluxes

are detected. It shows a section of axoplasm bounded on one side by the membrane and on the other by the porous dialysis capillary containing aequorin. Curve 1 is for the initial condition where the [Ca] of seawater is 3 mM and the $[Ca]_i$ of axoplasm is 30 nM. The system is in a steady state, and the aequorin glow is constant with time. A change to 6 mM Ca seawater produces a net inward flux of Ca which is rapidly reflected in a new value for $[Ca]_i$ just inside the membrane and a gradient in [Ca] across the axoplasm to the porous capillary (curve 2). The magnitude of $[Ca]_i$ just inside the membrane depends on the magnitude of the net Ca flux and on the speed with which axoplasmic buffering takes up most of the entering Ca. At infinite time, axoplasmic [Ca] rises to a value such that efflux

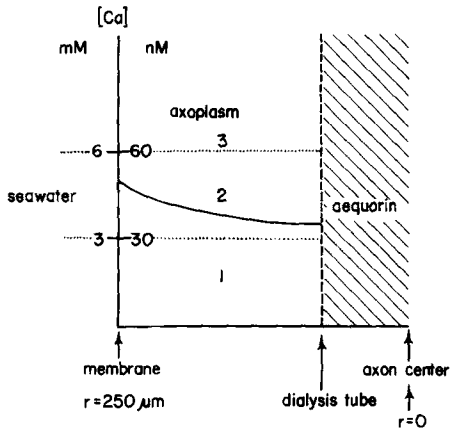

FIGURE 4. [Ca] in axoplasm as a function of distance (1) at zero time when the axon is in a steady state with $[Ca]_o$ = 3 mM, (2) at an intermediate time, t, after a change to $[Ca]_o$ = 6 mM, and (3) at $t = \infty$ after the change to $[Ca]_o$ = 6 mM. The aequorin at the center of the axon senses a far smaller change in [Ca] than that occurring at the membrane.

and influx are in balance, hence the gradient in [Ca] disappears and light emission is now enhanced, but is constant with time (curve 3). While the axoplasm is undergoing the transition from curve 1 to curve 3, aequorin light emission will be increasing with time. The slope of such a curve will not necessarily reflect the net flux at the membrane since, even when there is a $[Ca]_i$ at the membrane such that there is no net flux, some time will be required to abolish the Ca gradient across the axoplasm.

Calcium Net Flux

In the present study two ways of inducing a net flux were used: (*a*) the immersion of axons in seawaters containing 37 mM external calcium. A previous study showed that intact axons can maintain a constant Ca_i at 3 mM external calcium, but that they gain or lose calcium in seawater with higher or lower $[Ca]_o$; alternatively, (*b*) immersion of axons in low-$[Na]_o$ seawater. Previous work has defined the effect of such solution upon both influx and efflux (Baker et al., 1969; Blaustein and Hodgkin, 1969). This information, combined with the analytical data on total calcium already presented, can be used to make estimates

of the net calcium flux to be expected under various conditions, and of the ability of aequorin confined to a dialysis capillary to detect such net fluxes. These calculations are presented below.

In a previous study with aequorin (DiPolo et al., 1976) it was shown that axons in 10 Ca seawater exhibited an increase in resting glow with time, while in 3 Ca seawater, light emission was time independent. Fig. 5 A confirms these previous findings and allows one to measure changes in aequorin glow with time. The preceding section of this paper confirms these findings by showing that the Ca content of axons is in virtually a steady state in 3 Ca, while in 10 Ca seawater *Loligo* axons gain 64 fmol/cm² s. Hodgkin and Keynes (1957) and, more recently, Baker and McNaughton (1976) have shown that normal Ca influx from 10 Ca seawater is 100 fmol/cm² s and that Ca influx is linear with Ca_o of seawater. It is therefore possible to calculate that Ca influx and efflux are in balance in 3 Ca seawater at 36 fmol/cm² s. It is also possible to obtain net fluxes at other [Ca] in seawater by noting that Ca influx is linear and that efflux, for a fresh axon, should be constant and is taken as 40 fmol/cm² s. Table III makes this compari-

TABLE III

A COMPARISON OF AEQUORIN GLOW WITH NET Ca FLUXES

(Na) seawater [Ca]	Initial net Ca flux	Ca influx	Ca efflux	Observed slope aequorin glow/time*
mM	*fmol/cm² s*			*pA/min*
3	−7	33‡	40	−2.6
10	64§	100‡	40	+5.0
37	330	370‡	40	+14.0

* From Fig. 5 A.
‡ From Hodgkin and Keynes, 1957.
§ From Table II.

son by extrapolating from experimentally measured net flux values in 10 Ca seawater, and from measured Ca influx in this solution, to influx values for 3 Ca and 37 Ca seawater (if one assumes a constant initial value for Ca efflux of 40 fmol/cm² s). Included are measured slopes of aequorin glow vs. time (in picoamperes/minute) for the experimental record shown in Fig. 5 A. Since the mean resting glow of aequorin in fresh axons is 500 pA (Table IV), the detection of −2.6 pA/min in 10 min means observing a 5% change in light emission over this period of time. It would appear conservative, therefore, to claim that in a normal axon a net flux of 10 fmol/cm² s can be detected in 10 min by using aequorin, or that a flux of 2 fmol is detectable in 1 h.

Axons treated with CN or FCCP undergo a loss of mitochondrial buffering (Brinley et al., 1977) so that their sensitivity to net flux is now not a change in Ca_i that is 1/1,000 of the net Ca flux, but close to 1/20 of the net flux. The detection sensitivity of aequorin for Ca net fluxes in such inhibitor-treated axons is thereby increased from 10 fmol/cm² s to 0.2 fmol/cm² s.

Net Ca Fluxes in Low $[Na]_o$ Solutions

The effect of changes in the [Na] of seawater on $[Ca]_i$ is shown in Fig. 5 B; the axon was under observation for 24 h (recording between 16 and 19 h is omitted).

The record can be analyzed as follows. After 2 h of recording where the [Ca] of seawater was changed, the axon was surrounded with 3 Ca (choline) seawater for 3 h. The level of light emission rose 12-fold during this treatment and showed no signs of saturating. Recovery to resting glow levels took about 3 h, although initial recovery was rapid when 3 Ca(Na) seawater was applied. At hour 14 of the record, stimulation in 37 Ca(Na) seawater produced an essentially normal re-

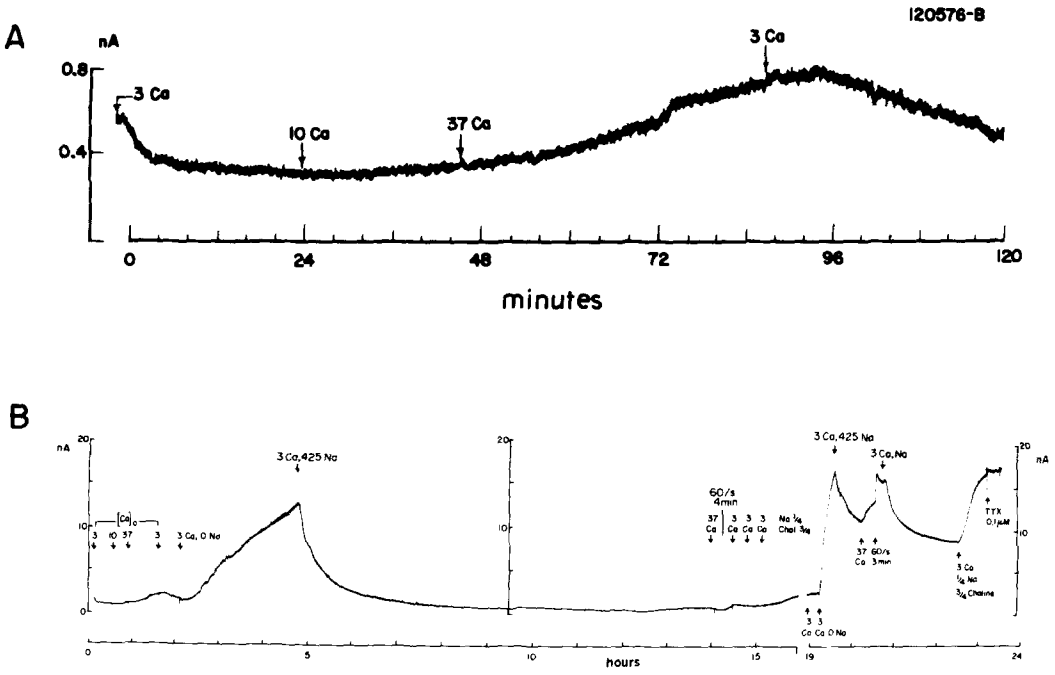

FIGURE 5. A, Changes in slope produced in a normal axon when the [Ca] of seawater is varied between 3 and 37 mM. B, Same axon shown over a recording period of 24 h. The trace is interrupted between 16 and 19 h, and the axon was in 3 mM Ca (Na) seawater for 2 h before commencement of recording.

sponse, but a second treatment with 3 Ca (choline) seawater at 19 h showed a markedly more rapid response than that measured earlier. Additionally, the response to stimulation in 37 Ca seawater was also 10 times greater. Finally, the response to 25% Na (choline) seawater was TTX insensitive. The differing sensitivity of the axon to choline seawater at 2 h and 19 h is difficult to ascribe to an altered leak of Ca since $[Ca]_o$ was the same in both cases. It might be thought to be the result of an increased $[Na]_i$ since Ca influx is highly dependent on this variable. However, the Ca concentration change resulting from stimulation, which is not thought to depend on Na_i, was also enhanced so that it would appear most likely to ascribe this change to a deterioration of axoplasmic buffering after long periods. A change in buffering cannot be expected to affect levels of $[Ca]_i$ when fluxes are in balance, but a decrease in buffering will enhance the response of aequorin to an applied Ca load.

Effect of $[Na]_0$ on $[Ca]_i$

Several studies have indicated that reducing Na_0 increases Ca influx and presumably results in a new steady state for both free and total Ca in axoplasm. The experiments described below were undertaken to study directly the relation between Na_0 and the steady-state level of ionized Ca at the core of the axon.

A number of axons have been examined in 180 mM Na, 3 Ca seawater (40% Na), and in no case is there a rise in Ca_i with time. On the other hand, 90 mM Na, 3 Ca seawater invariably induces a rise in Ca_i with time, as do all lower concentrations of Na in seawater. A representative experiment is shown in Fig. 6. As shown in the figure, reducing the [Ca] of seawater to 0.5 mM allows one to

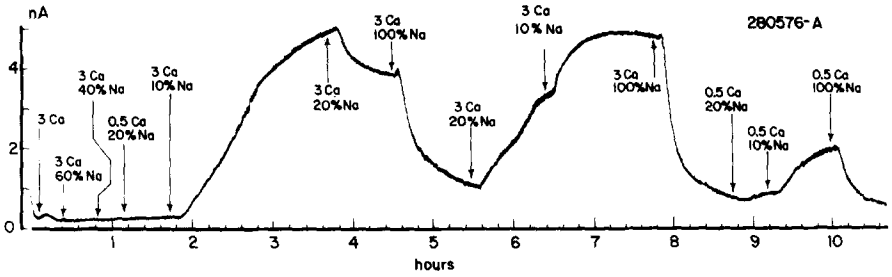

FIGURE 6. Aequorin light emission as a function of time is shown for a normal axon subjected to the various changes in $[Na]_o$ and $[Ca]_o$ shown on the figure. 100% Na = 450 mM.

apply 20% Na with very little effect on $[Ca]_i$. About a 30-fold increase in resting glow can be extrapolated from the record where 3 Ca (10% Na) seawater was applied, and a steady level of 20 times resting glow for 3 Ca (20%) Na seawater is also shown. Recovery towards the base line was rapid in 3 Ca (100% Na) seawater, and a reapplication of 3 Ca (10% Na) seawater led to a 25-fold increase in resting glow. A final application of 0.5 Ca (10% Na) seawater led to an increase in resting glow about nine times normal. The application of large Ca loads by reducing the Na in seawater to 10% takes about 2 h to reach a plateau.

Effect of Membrane Potential on $[Ca]_i$

In dialyzed squid axons, hyperpolarization is known to increase Ca efflux (Mullins and Brinley, 1975) and depolarization to decrease Ca efflux (Blaustein et al., 1974; Mullins and Brinley, 1975). In injected axons, depolarization has been shown to increase Ca_o-dependent Na efflux (hence presumably Ca influx), while hyperpolarization appeared to have only a small effect on Ca efflux (Baker and McNaughton, 1976). This latter finding may reflect the extreme lability of $[Ca]_i$, since an increased Ca efflux may deplete the axon of ionized Ca before flux measurements can be made. At any rate, the experimental information presently available suggests that depolarization of the membrane should increase Ca influx, decrease Ca efflux and hence result in an increase in Ca_i. An experiment to test these predictions is shown in Fig. 7. The axon had a normal resting glow in seawater containing 3 Ca, 360 Na, 100 choline seawater. This increased 4.6-fold upon the application of 100 mM K seawater (360 Na), and it

recovered to control values when the membrane was allowed to repolarize in seawater with a normal [K].

Apyrase-Injected Axons

A number of recent publications (DiPolo, 1973, 1974, 1976; Baker and McNaughton, 1976) have shown that ATP has an effect on Ca efflux. In the present study, experiments were done to determine the effect of ATP upon internal ionized Ca as well as the ability of an axon to buffer and to recover from an imposed Ca load. The microinjection of apyrase was used as a convenient way of reducing axoplasmic ATP to low levels without, presumably, compromising the Ca buffering function of the mitochondria.

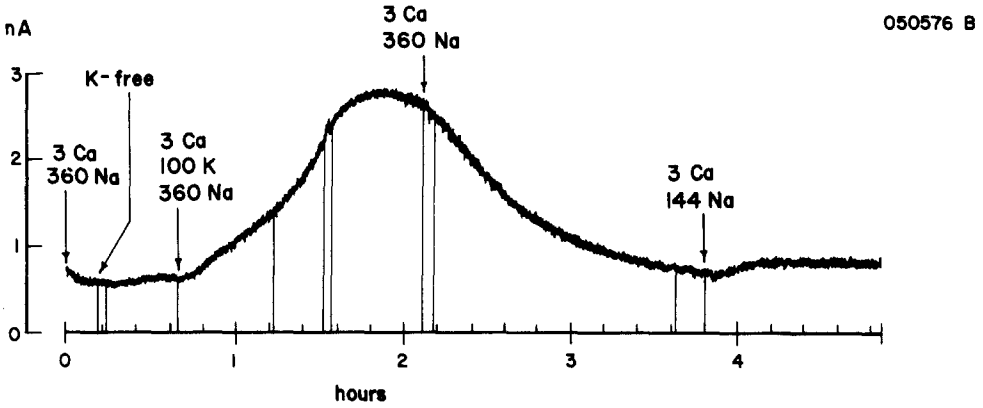

FIGURE 7. Response of an axon to 100 mM K seawater applied for close to 1.5 h, leading to a fivefold increase in aequorin glow and the subsequent recovery of the axon, when 100 mM K is removed, to levels of resting glow. The legend "K-free" denotes the application of K-free Na seawater for 10 min with a return to 3 Ca (Na) seawater.

Fig. 8 A shows the response of an apyrase-injected axon to a change from 3 Ca (Na) seawater to seawater with 37 mM Ca. The initial resting glow in 3 Ca was 0.23 nA and was stable with time; this rose in 37 Ca to a value of 2 nA and recovered to virtually its initial value in 3 Ca seawater. The change in glow is 10-fold and the change in Ca_o 12-fold—reasonable agreement with the idea that Ca_i must rise sufficiently so that Ca efflux balances Ca influx at a new steady-state value. Subsequent treatment of the axon with 33% Na seawater (3 Ca) led to only a small increase in ionized Ca, and stimulation in 37 Ca seawater gave an increase in Ca_i that is comparable to that observed in normal axons. A change to choline (0 Ca) seawater led to a decline in ionized Ca, indicating that net Ca extrusion is possible in choline seawater. The analytical studies of Ca in axoplasm (Fig. 2) show that Ca loss in Ca-free seawater is very small over a period of 4 h, while the above effect of choline takes place in 30 min. The application of Ca-free (Na) seawater brings Ca_i down to levels slightly less than the initial resting glow. An experiment of this sort leads to the conclusion that ionized Ca can be increased 10-fold in an axon with very low $[ATP]_i$, and that the membrane pump can reduce ionized [Ca] to normal values after the load.

Another experiment is illustrated in Fig. 8B. The application of 40% Na seawater was without effect on the resting glow of the axon; this effect is identical to that observed in normal axons. The application, successively, of 20%, 10%, and 0% Na seawater led to increases in Ca_i which are many fold

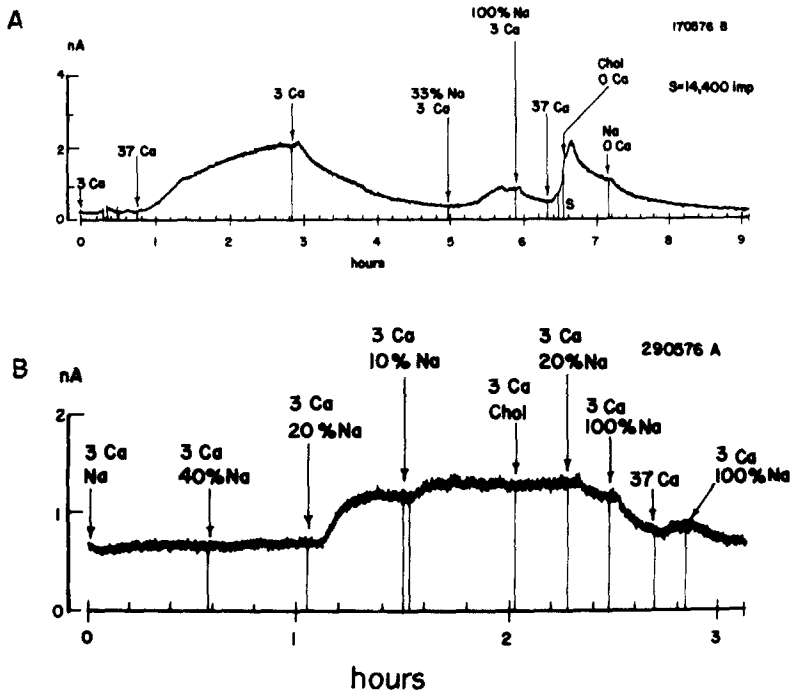

FIGURE 8. A, Apyrase-injected axon. It shows a virtual steady state of glow of the axon in 37 mM Ca seawater and a value about 10 times the initial resting glow, followed by a complete recovery in 3 mM Ca seawater. One gets a small rise in $[Ca]_i$, followed by a leveling off in 1/3 Na seawater and, in the total absence of external Ca, Na seawater is capable of bringing the resting glow down to low levels. B, Another apyrase-injected axon, showing that in 20% Na seawater the glow rises and levels off. 10% Na gives an additional rise while 0% Na gives no further change. The axon does recover, however, to its initial value of resting glow at the end of the trace. The behavior in 3 mM Ca (choline) seawater is enormously different from the behavior of a normal axon in this medium.

smaller than those observed in normal axons. A normal axon shows a 12–15-fold increase in ionized Ca and no sign of a steady state after 2 h in choline seawater, while an apyrase-injected axon shows a 1.7-fold increase in Ca_i that is essentially in a steady state in 12 min. Since the principal effect of choline seawater is to increase Ca influx greatly (Ca efflux is affected but quantitatively contributes little to the net flux), these observations suggest that in the absence of ATP the influx is not increased by choline seawater. A further conclusion is that 3 Ca (choline) seawater is capable of maintaining a steady state with respect to ionized Ca.

The somewhat surprising findings of Fig. 8 A and B might be explained if one could assume that the buffering capacity of Ca by axoplasm has somehow been

increased by the removal of ATP from the axon. This seems unlikely, since mitochondrial buffering is known not to depend on ATP in the presence of adequate substrate (Brinley et al., 1977) and because the response of an apyrase-injected axon to both stimulation and 37 Ca (Na) seawater is normal.

The experiment shown in Fig. 9 was done to look at other factors involved in Ca homeostasis. Stimulation gave the usual response found in normal axons,

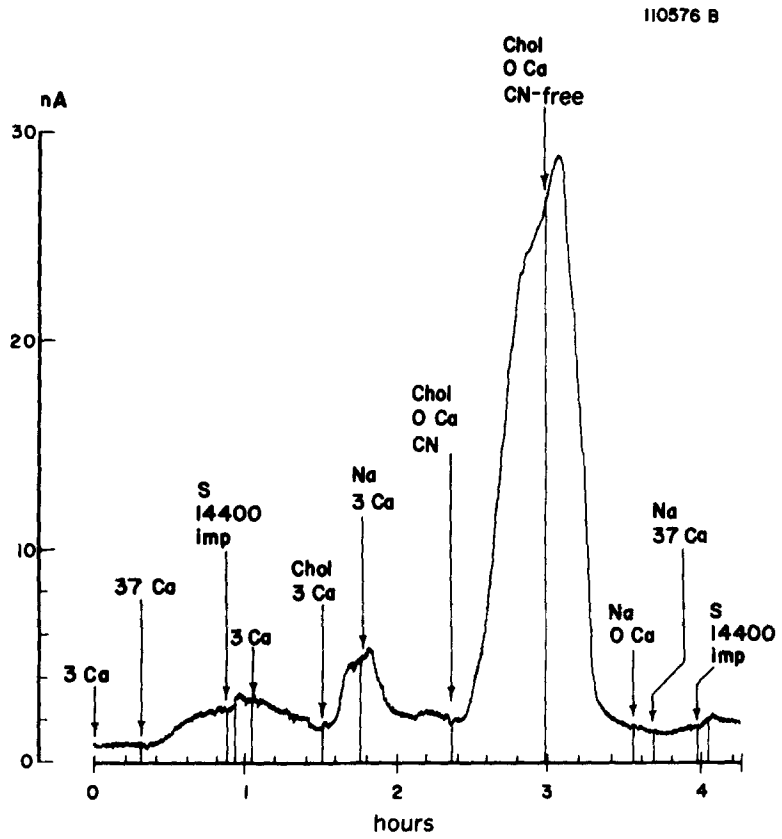

FIGURE 9. Axon preinjected with apyrase which shows appropriate increases in resting glow in 37 mM Ca seawater, and recovery after return to 3 mM Ca seawater. The most important feature of this illustration is a demonstration that at about 2.2 h, when CN was applied, there was a large and virtually immediate increase in glow instead of a wait before the effects of CN would be manifest. The CN effect was fully reversible and the axon recovered its resting glow.

and 3 Ca (choline) seawater gave a 2.2-fold increase in ionized Ca, while 3 Ca (Na) seawater caused a reduction in Ca_i. A change to 0 Ca (choline) seawater + 2 mM CN gave, within the mixing time of the flow system, an immediate and large increase in resting glow that was promptly reversed upon the removal of CN. The immediate effect of CN is understandable on the basis that in the absence of ATP, a block of electron transport in mitochondria causes an immediate release of the calcium load induced by the prior exposures to low sodium solutions. The removal of CN causes an immediate uptake of Ca, presumably by the mitochon-

dria. Stimulation carried out at 4 h shows that buffering of axoplasm is unchanged over that observed at about 1 h. Additional evidence that axons can maintain a near normal Ca_i in the virtual absence of ATP_i is provided by Table IV, which shows that the initial resting glow of apyrase-injected axons is not greater than that of control axons.

TABLE IV

IONIZED [Ca] IN NORMAL AND APYRASE-INJECTED AXONS
3 Ca (Na) SEAWATER, T = 15°C

Normal axons (15 min after mounting)		Apyrase-injected axons (30 min after injection)	
Axon ref	Aequorin glow	Axon ref	Aequorin glow
	nA		*nA*
030576	0.60	270576	0.52
040576A	0.40	170576B	0.23
150576	1.00	140576B	0.54
180576B	0.42	120576A	0.95
180576A	0.45	110576B	0.38
280576A	0.38	110576A	0.40
250576B	0.60	100576A	0.28
170576A	0.45	060576A	0.40
130576	0.30	290576A	0.70
300576B	0.60	050576B	0.60
300576A	1.20		0.50 ± 0.07
290576B	0.40		
260576	0.32		
250576A	0.28		
240576A	0.20		
230576B	0.40		
230576A	0.22		
220576B	0.20		
220576A	0.22		
210576	0.33		
140576A	0.20		
020676A	1.00		
	0.46 ± 0.06		

~0.5 nA = 20 nM [Ca].

Mitochondrial Inhibitors

It has been recognized for some time that mitochondria are capable of accumulating Ca and may serve an important regulatory function inside a nerve fiber. Earlier observations (Blaustein and Hodgkin, 1969) showed that it was possible to increase Ca_i some 30-fold when an axon was treated with CN for times long enough for all ATP to be used up. These axons had, however, been isolated for some time and had 400 μmol Ca/kg axoplasm. More recently (DiPolo et al., 1976), it became clear that in freshly isolated axons the ionized Ca rose by at most a factor of 2 when they were subjected to CN seawater, in the absence of Ca_o, while axons deliberately loaded with calcium and subsequently tested with CN always gave a large release of calcium.

The main purpose of the experiments performed with mitochondrial inhibitors was to show that in the presence of such substances, a squid axon is capable

of regulating its internal [Ca] via the membrane pump, even though it has undergone a substantial loss of Ca buffering. The question of axoplasmic buffering is the subject of another report (Brinley et al., 1977), and in the present context the experiments were designed to show that axoplasmic Ca regulation is possible in the presence of CN or FCCP, the latter a hydrogen ionophore.

To confirm the previous findings of DiPolo (1976) an axon was treated with 0 Ca (choline) seawater as shown in Fig. 10 A, and the light emission of aequorin

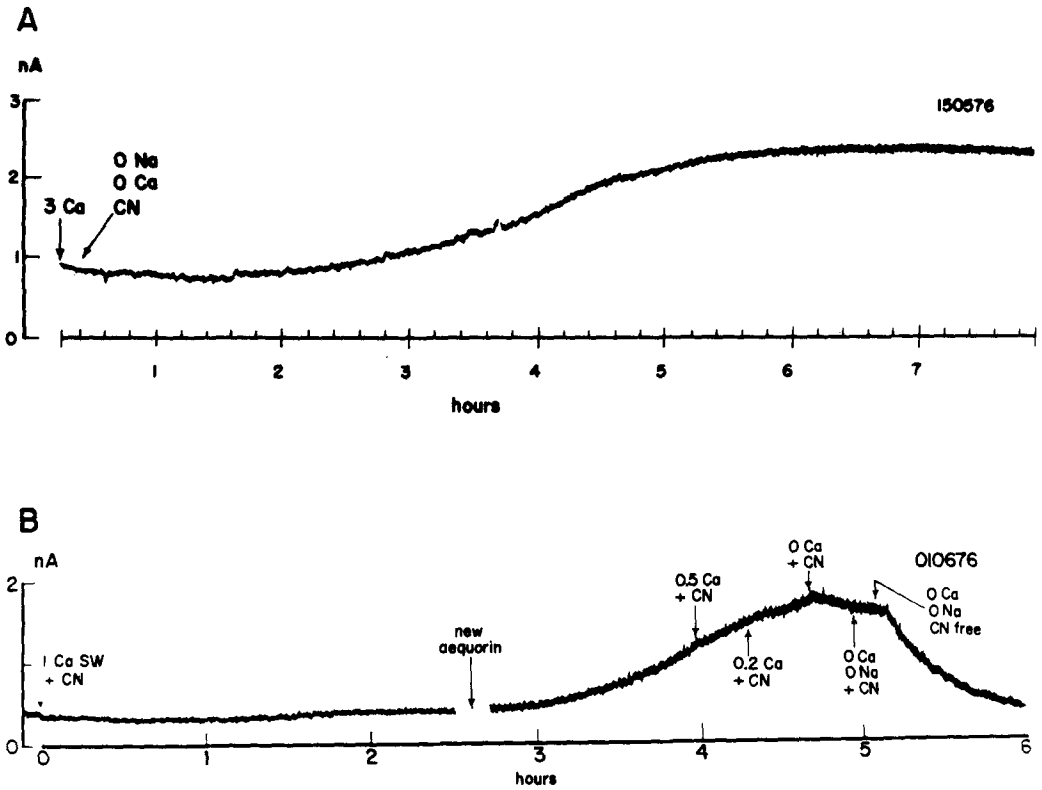

FIGURE 10. A, Normal axon with a resting glow in 3 mM Ca and the almost immediate transfer of this axon to 0 Na, 0 Ca CN seawater. After about 3 h, the resting glow slowly begins to rise and about doubles in 6 h. This shows the magnitude of releasable Ca after a long CN immersion. B, Experiment similar to A, except that the axon was kept in 1 mM Ca (Na) seawater with 2 mM CN for 5 h and the CN was then removed.

followed. The result was a doubling in the aequorin glow that required about 4 h for half completion. Ca-free seawater was used to insure that no Ca entered the fiber, and choline seawater was employed to minimize Ca efflux. A second axon (Fig. 10 B) was kept in 1 mM Ca (Na) seawater throughout its exposure to CN in order to demonstrate that it is not necessary to use Ca-free solutions to control the ionized Ca in the presence of CN. The release of Ca from mitochondria follows essentially the same time course as the axon in Fig. 10 A, and this release

was uninfluenced by reductions in the [Ca] of seawater to 0.5 and 0.2 mM. The change to 0 Ca seawater apparently coincided with the peak release of Ca, and since the seawater contained full Na, some Ca pumping was to be expected. Note that the removal of CN brought the ionized [Ca] down to its initial value but with a time constant of the order of 30 min, as compared with 1/6 of this value in Fig. 9 where the ionized [Ca] was quite high. This suggests that the rate of mitochondrial uptake of Ca at a Ca_i of ~90 nM is quite slow.

Another mitochondrial inhibitor used was FCCP. This ionophore makes it impossible for mitochondria to maintain their normal function, and the result shown in Fig. 11 A is a virtually immediate release of stored Ca upon application of the inhibitor. The change in ionized [Ca] is threefold in all cases where this inhibitor was used as compared with a twofold change for CN. This discrepancy is probably accounted for by the fact that it may take 4 h for a CN effect, during which time some appreciable Ca efflux must have been expected to occur. This view is reinforced by noting that in Fig. 11 A at 4 h the aequorin glow is twice the initial resting glow.

A more extensive study of the ability of FCCP-treated axons to regulate Ca_i is shown in Fig. 11 B. Here the axon was in 3 Ca (Na) seawater when FCCP was applied, and the usual threefold increase in aequorin glow ensued. Subsequently, there was a clear rise in glow with time that changed to a decline in glow when 1 Ca (Na) seawater was applied. A change to 60% (Na) 1 Ca seawater appeared to produce a steady Ca_i, while 40% (Na) 1 Ca seawater gave a slope very similar to 3 Ca, 100% Na seawater. A final change to 20% (Na) 1 Ca seawater led to a very large increase in aequorin glow, and one that was not reversed by 100% Na seawater applied for 1 h.

A comparison of this figure with Fig. 6 where a normal axon was treated with 20% Na, 3 Ca seawater shows that the slope was 65 pA/min. For 1 Ca seawater this would be 22 pA/min as compared with 130 pA/min for Fig. 11. These slopes differ by a factor of 6, but this does not necessarily imply that the net Ca flux is larger in FCCP-treated axons. Rather, the buffering of Ca has been decreased so that a smaller net flux results in larger changes in Ca_i (see Brinley et al., 1977).

DISCUSSION

Estimates of Ca Net Flux in Na-Free Solutions

Measurements of Ca influx from mixtures of Na and Li seawater have been made by Baker et al. (1969), and Fig. 11 of their paper shows that Ca influx declines linearly with increases in Na_o and reaches values the same as in 450 mM Na when Na_o is 220 mM. Since we have observed no net Ca flux when Na_o is 180 mM, we can calculate net fluxes in choline as follows: (Ca influx in choline, 3 Ca) $\times$ $[(180 - [Na]_o)/180]$ will give values for the influx promoted by low [Na], and to this must be added the Ca influx in 3 Ca (Na) seawater. Since from Table II the net flux into axons in 10 Ca (choline) seawater is 8,300 fmol/cm^2 s, and since efflux from an axon with a normal $[Ca]_i$ is <1% of this, the value is really an influx. Calculated and observed changes in $[Ca]_i$ are shown in Table V.

Since Ca influx varies as the square of $[Na]_i$, and $[Na]_i$ can be expected to vary from axon to axon, the agreement between calculated fluxes and observed

changes in $[Ca]_i$ is reasonable. No allowance has been made in the above calculations for the fact that Ca efflux declines in an intact axon when Na-free solutions are applied. The effect is not large; Baker and McNaughton (1976)

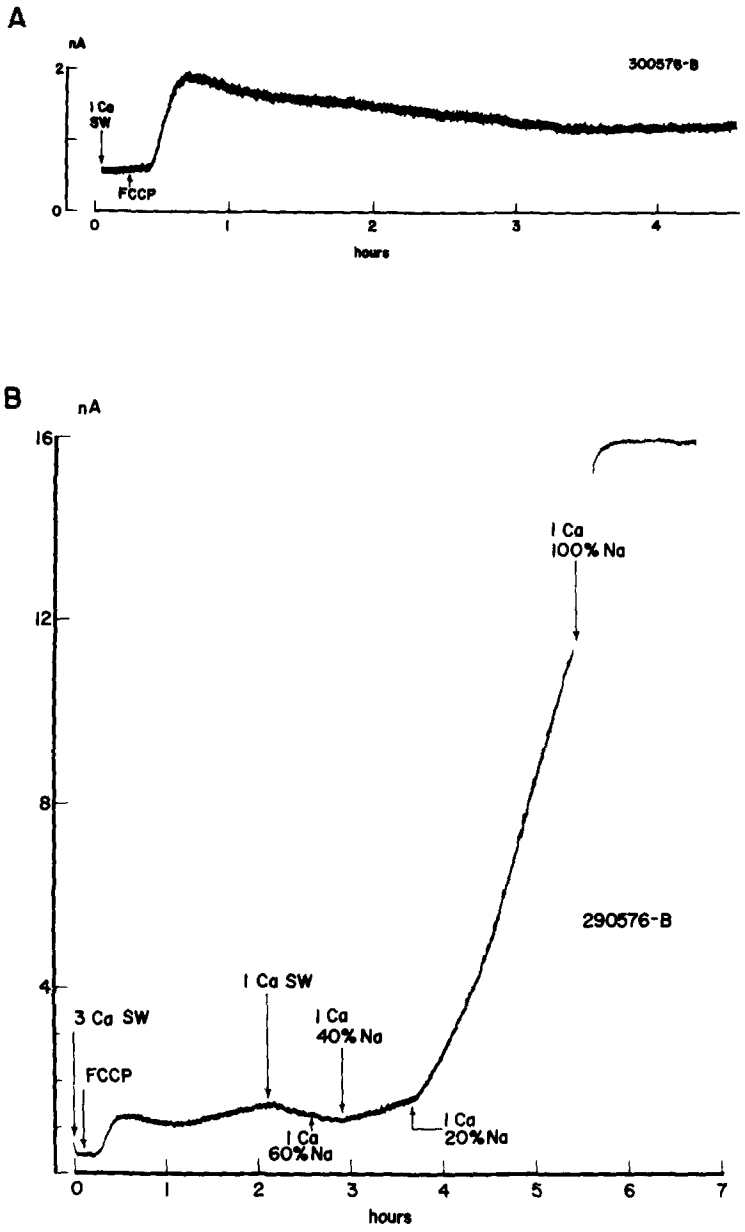

FIGURE 11. A, Axon is treated with FCCP in 1 Ca (Na) seawater. The response is a rapid release of Ca from internal stores and the extrusion of some of this Ca over 3 h. Final $[Ca]_i$ is about twice the initial concentration. B, Another FCCP-treated axon showing a gain in $[Ca]_i$ in 3 Ca, a loss in 1 mM [Ca], and a large increase in 1 mM Ca 20% Na seawater.

show a 33% decline in efflux in Na-free solutions. Application of such a correction would have the effect of increasing the calculated $[Ca]_i$ required for flux balance.

The main point in this tabulation is that for normal axons the calculated increases in $[Ca]_i$ in the steady state are in rough agreement with those experimentally measured, while for apyrase-injected axons the values are of the order of 10 times too low. This point is discussed further below.

Effect of Ca_o on Normal Axons

Ca can enter an axon by a variety of pathways. For the normally polarized axon we can class the pathways as: (*a*) diffusion or leak; (*b*) exchange for Na_i; (*c*)

TABLE V

CALCULATED NET Ca FLUXES AND OBSERVED CHANGES IN $[Ca]_i$ IN Na-CHOLINE 3 mM Ca MIXTURES

$[Na]_o$		Ca influx	Calculated increase in $[Ca]_i$ for flux balance	Normal axons		Apyrase axons	
				Axon reference	Observed increase in $[Ca]_i$	Axon reference	Observed increase in $[Ca]_i$
%	*mM*	*fmol/cm² s*	*-fold*		*-fold*		*-fold*
0	0	2,500	76	180576B	18	110576B	2.5
				120576B	≫12	290576A	2.0
				190576A	10		
				210576	5		
10	45	1,900	58	280576A	25	290576A	2.0
				280576A	31		
20	90	1,300	40	280576A	11	290576A	1.7
				210576	3	270576	3.0
33	150	400	12	140576A	1	170576B	3.0
						140576B	2.0*
40	180	33	–	280576A	1	290576A	1.0
				260576	1		
100	450	33	–				

* Na_i elevated ~40 mM.

exchange for Ca_i. Pathway (*c*) in its conventional interpretation cannot result in net Ca flux and so need not concern us. Pathways (*a*) and (*b*) could, in principle, be distinguished by making Ca influx measurements in axons with $[Na]_i = 0$ as compared to axons with a normal $[Na]_i$, but such measurements have not been done. A study by Rojas and Taylor (1975) involving the measurement of Ca influx into perfused axons without Na in the perfusion fluid showed that Ca influx from 10 Ca (Na) seawater was 10 fmol/cm² s, a value only 10% of that of intact axons. Such measurements were made with F as the principal intracellular anion. More relevant may be the findings of Fig. 5 A,B which show that Ca influx in an apyrase-injected axon is readily increased by a change of $[Ca]_o$ from 3 to 37 mM, while a change from Na to choline seawater has only a small effect. This suggests that an appreciable fraction of the Ca influx may be a diffusion or leak.

In some FCCP-treated axons, the $[Ca]_i$ stabilizes at a value about twice the resting glow if $[Ca]_o$ is 1 mM in (Na) seawater, while in others (Fig. 11 B) there is a

decline in $[Ca]_i$ followed by an increase in 3 Ca seawater. A reasonable interpretation of these findings is that since, in the presence of mitochondrial inhibitors, the sensitivity of aequorin to net Ca fluxes is effectively increased by a factor of ~50, minor changes in $[Na]_i$ are critical in determining the Ca_o for flux balance.

While buffering by mitochondria appears to be constant in axons not treated with inhibitors, an exception appears to be the axon shown in Fig. 5 at times greater than 20 h where the response to stimulation is a much greater increase in $[Ca]_i$ than was exhibited by this same axon at an earlier time. Baker et al. (1967) have also noted that axons kept for long times had a virtually normal resting glow but responded to stimulation with a greatly enhanced light output; they characterized such axons as "sensitive." The cause of this sensitivity would appear to be the loss of axoplasmic buffering, presumably because of the depletion of substrates for mitochondrial metabolism without a corresponding impairment of the membrane Ca pump.

Effect of Lowered $[Na]_o$ on $[Ca]_i$

In contrast to the findings of changes of Ca_o on Ca net flux, changes in $[Na]_o$ cannot be expected to change the leak of Ca into an axon but only to activate to a greater or lesser extent the influx of Ca in exchange for Na_i. A significant finding in this investigation is that $[Ca]_i$ remains constant if Na_o is reduced from 450 mM, its normal value in seawater, to 180 mM (40% Na). If the energy available in the Na gradient across the membrane is to energize the efflux of Ca, then $nzF(E_{Na} - E_m)$ must be equal to or greater than $zF(E_{Ca} - E_m)$ where n is the coupling ratio Na/Ca. For an $[Na]_i$ of 40 mM, E_{Na} in seawater is +60 mV, and with the usual value of E_m of −60 mV, the Na gradient term is +120 mV. A previous investigation (DiPolo et al., 1976) has measured a value for $[Ca]_i$ of 30 nM and for a steady state, a value of $[Ca]_o$ of 3 mM or a ratio of [Ca] across the membrane of 10^5. This yields a value for E_{Ca} of +145 or a value for the Ca gradient $z(E_{Ca} - E_m)$ of +410 mV. The coupling ratio Na/Ca must clearly be 4 or greater; this yields +480 for the Na gradient. At an $[Na]_o$ of 180 mM, E_{Na} is now 38 mV or the Na gradient is 4 × 98 mV or 392 mV as compared to an energy demand of 410 mV. These two values are sufficiently close to allow the conclusion that Ca extrusion from the Na gradient can proceed at an $[Na]_o$ of 180 mM. Lower values of $[Na]_o$ will clearly be insufficient to support the usual value for $[Ca]_i$, and the experiments reported confirm this prediction. A factor not allowed for in the calculations presented above is the extent to which choline (used as a diluent for Na seawater) has the ability to extrude Ca. The choline gradient is far larger than the Na gradient owing to the absence of this compound inside the axon. In a study with dialyzed axons, Brinley et al. (1975) showed that when Ca_i was low, and in the absence of ATP, Ca efflux was the same in Na and choline seawater. This does not address directly the effects that choline might have on Ca influx, but does suggest that this ion has some ability to extrude Ca from inside the axon. Since the specificity of the Na-Ca exchange system has not been investigated with respect to other ions that might serve as replacements for Na, some caution ought to be exercised in assuming that ions such as choline, Li, or Tris have no ability to serve as Na substitutes. In dialyzed axons with a high $[Ca]_i$ (1 μM or greater) the application of choline seawater

reduces Ca efflux by 99% or more (Blaustein and Russell, 1975); the residual efflux, however, is more than enough to support Ca flux balance. In intact axons, injected with ^{45}Ca, the efflux of Ca is elevated and choline or Tris seawater reduces the Ca efflux by about 1/3 while the residual efflux is more than enough to support normal values of Ca efflux.

Effect of Depolarization on $[Ca]_i$

There is an extensive literature on the effects of membrane potential changes on Ca influx. Hodgkin and Keynes (1957) found that repetitive stimulation increased Ca influx, and that depolarization of the fiber with 200 mM KCl seawater increased Ca influx fivefold. Baker et al. (1971) showed that part of the response to stimulation was TTX sensitive and part was not. They termed this TTX-insensitive response to depolarization the "late channel" for Ca entry. Mullins and Brinley (1975) showed that hyperpolarizing squid axons increased Ca efflux, and Blaustein et al. (1974) and Mullins and Brinley (1975) showed that depolarization decreased Ca efflux. Baker and McNaughton (1976) showed that in intact axons there was a large increase of the Na efflux, in response to depolarization, that was Ca_o dependent (implying an increase in Ca influx coupled to Na efflux) but that there was a smaller response of Ca efflux to hyperpolarization.

All of the literature cited above is in general agreement with the idea that depolarizing a squid axon increases Ca influx and decreases Ca efflux, so that the expected response of $[Ca]_i$ is an increase. It is important to recognize that if Ca movement depends on the Na electrochemical gradient, then the terms for electrochemical potential of Na and of Ca contain E_m. A theoretical argument based on thermodynamics (Mullins, 1976) has suggested that flux balance is obtained when $nzF(E_{Na} - E_m) - zF(E_{Ca} - E_m) = 0$. With the coupling ratio n = Na/Ca = 4, this reduces to $2F(2E_{Na} - E_m - E_{Ca}) = 0$ and clearly indicates a dependence of flux balance on membrane potential.

In the experiment shown in this report, the expected effect of 100 mM K seawater is a 20-mV depolarization of the resting potential. This should decrease Ca efflux by exp 20/25 and increase Ca influx by a like amount, so that the expected change in Ca_i is exp 40/25 or 4.95. The observed increase in Ca_i in the steady state is fivefold, or in reasonable agreement with calculation.

Effect of CN and FCCP on $[Ca]_i$

Experiments with 2 mM CN show that in normal axons one observes after several hours a release of Ca that amounts to a doubling of the $[Ca]_i$, or an increase of 30 nM. Analytical measurements show that total axoplasmic Ca is 60 μM so that only 0.5% of this is apparently in a releasable form in mitochondria (the actual release is about 10% of the total Ca, but the major fraction of this is taken up by nonmitochondrial buffers). Another finding with CN is that it is possible to maintain a virtually normal level of ionized Ca in axoplasm in the presence of this inhibitor at times when the ATP concentration is low enough to allow mitochondria to release their accumulated Ca, thus reinforcing the conclusions made as a result of apyrase experiments.

The application of FCCP to an axon results in an immediate release of mitochondrial Ca, since this inhibitor effectively abolishes the ability of mitochondria to carry out either oxidative phosphorylation or Ca accumulation. Our observations show that the $[Ca]_i$ of a fresh axon rises by 60 nM when this substance is applied, and that this invariably declines to about 40 nM during the 1st h of application of FCCP. Over periods of 4 h it is, however, possible to stabilize Ca_i at values near normal if the Ca_o is reduced from 3 mM to 1 mM, mainly, we believe, because increases in Na_i and consequent effects on Ca flux balance ensue as a result of the low level of ATP in the fiber. Furthermore, since the application of inhibitors results in an immediate loss of mitochondrial buffering, the detection sensitivity of aequorin is increased from 10 fmol/cm^2 s to one of 0.2 fmol/cm^2 s. With a normal flux of 30 fmol/cm^2 s in 3 Ca seawater, a 1% imbalance in flux can be detected as compared with a 30% flux imbalance in normal axons. Because CN-treated axons must be gaining Na, with a consequent decrease of the electrochemical gradient of Na across the membrane, one should ideally be decreasing the Ca electrochemical gradient to compensate for this effect. We have, in fact, found it possible to decrease Ca_o to 1 mM in seawater and have a flux balance for times of the order of 4 h. For longer times (6–8 h) the Ca_o for flux balance appears to lie between 0 and 0.4 mM.

Ability of ATP-Depleted Axons to Extrude Net Calcium

Experiments with apyrase-injected axons show clearly that net extrusion of ionized calcium after an imposed calcium load can occur in the near absence of ATP. Although the calcium-buffering systems seem to be largely intact in this situation, and a mitochondrial component cannot be entirely ruled out, it appears that the steady-state levels of ionized calcium resulting during loading and the return of ionized calcium to resting levels after the end of loading reflect membrane transport for the following reasons. The net load induced by sodium-free solutions had been shown to be about 4 μmol Ca per kg axoplasm h per mM Ca_o by direct analytical measurement. In the following paper, the mean increment in ionized calcium, for small loads, is shown to be about 1 nM/μmol/kg axoplasm of Ca load. For the loading shown in Fig. 8 B (3 mM Ca_o), the increase in ionized calcium would be about 12 nM/min. Since the resting light level is equivalent to about 20–50 nM ionized calcium, the twofold increase in light seen during the entire period in low or 0 sodium solutions (80 min) is clearly incompatible with a rise of ionized calcium anywhere near the calculated rate of 12 nM/min.

Even with generous allowances for experimental error in the estimation of calcium load and buffering capacity, it seems necessary to conclude that the steady states observed in apyrase axons reflect mainly the ability of calcium efflux to compensate for the increased influx. In view of the good evidence that calcium efflux in low ATP axons is not greatly affected by external sodium when the ionized calcium is in the physiological range (in none of these axons did the ionized calcium ever rise above two to three times normal), it seems simplest to conclude that the reduction in ionized calcium which occurs after return of the axons to sodium-containing seawater (with reduction of influx to normal levels) reflects persistence of the elevated efflux, thus producing a net extrusion of

calcium. This conclusion applies only in the ionized moiety; there is no direct evidence that the increment in total calcium induced by the load is similarly extruded, but such a change would require in effect that the affinity of axoplasmic buffers for Ca be enhanced by the absence of ATP.

Effect of ATP on Calcium Fluxes

An important observation from this investigation is that axons with low axoplasmic ATP concentrations maintain essentially normal $[Ca]_i$ in the presence of a physiological concentration of extracellular calcium. The evidence for these conclusions comes from the axons injected with apyrase which reduce axoplasmic ATP to low levels. Additional evidence comes from the cyanide-treated axons (e.g., Fig. 10), although in this case the internal ionized calcium rises somewhat, probably because the exposure to cyanide not only reduces axoplasmic ATP and increases Na_i but also impairs mitochondrial buffering so that a slight calcium flux imbalance is more obvious.

The estimates, given in the first section of the Discussion, that aequorin can detect a net calcium influx of about 10 fmol/cm^2 s, indicate that the influx of calcium cannot exceed the efflux by more than this amount in a low-ATP axon. Recent reports from several laboratories show that Ca efflux is reduced in the absence of ATP. Since $[Ca]_i$ has been shown to be constant in the absence of ATP, one concludes that a major fraction of the normal Ca influx requires ATP and is reduced in its absence. The fractional reduction in influx must have been comparable to the reduction in efflux which, under the conditions of the present experiments, must have been substantial. An accurate estimate of the ATP sensitivity of the Ca efflux in an intact axon is difficult to make because it depends strongly upon the internal ionized concentrations of both calcium and sodium. Published data on both injected and dialyzed axons (Baker and McNaughton, 1976; DiPolo, 1976; Blaustein et al., 1975) in which the ionized calcium was about 100 nM and the internal sodium 40–80 mM indicate that removal of ATP reduces calcium efflux by at least a factor of 3, and more recent data (DiPolo, unpublished observations) at physiological $[Ca]_i$ indicate that the reduction is even greater, perhaps a factor of 10–20. Although the obvious dependence of calcium efflux upon internal ATP and the inability of the nonhydrolyzable ATP analogues to substitute for ATP suggest that it functions as a hydrolyzable substrate (DiPolo, 1976), the marked dependence of Ca efflux upon what could be, on a thermodynamic basis, purely a coupled Na influx cautions against assigning any single simple role for ATP in calcium transport.

An obvious implication from the finding that Ca unidirectional fluxes are affected by ATP is that this substance could increase the rate at which an axon recovers from an applied Ca load.

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REFERENCES

BAKER, P. F., M. P. BLAUSTEIN, A. L. HODGKIN, and R. A. STEINHARDT. 1969. The influence of calcium on sodium efflux in squid axons. *J. Physiol. (Lond.)*. **200**:431–458.

BAKER, P. F., A. L. HODGKIN, and E. B. RIDGWAY. 1971. Depolarization and calcium entry in squid axons. *J. Physiol. (Lond.)*. **218**:709–755.

BAKER, P. F., and P. A. MCNAUGHTON. 1976. Kinetics and energetics of calcium efflux from intact squid giant axons. *J. Physiol. (Lond.)*. **259**:103–144.

BLAUSTEIN, M. P., and A. L. HODGKIN. 1969. The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol. (Lond.)*. **200**:497–527.

BLAUSTEIN, M. P., and J. M. RUSSELL. 1975. Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. *J. Membr. Biol.* **22**:285–312.

BLAUSTEIN, M. P., J. M. RUSSELL, and P. DE WEER. 1974. Calcium efflux from internally dialyzed squid axons: the influence of external and internal cations. *J. Supramol. Struct.* **2**:558–581.

BRINLEY, F. J., JR., and L. J. MULLINS. 1968. Sodium fluxes in internally dialyzed squid axons. *J. Gen. Physiol.* **52**:181–211.

BRINLEY, F. J., JR., S. G. SPANGLER, and L. J. MULLINS. 1975. Calcium and EDTA fluxes in dialyzed squid axons. *J. Gen. Physiol.* **66**:223–250.

BRINLEY, F. J., JR., T. TIFFERT, A. SCARPA, and L. J. MULLINS. 1977. Intracellular calcium buffering capacity in isolated squid axons. *J. Gen. Physiol.* **70**:355–384.

DE WEER, P. 1970. Effects of intracellular adenosine-5'-diphosphate and orthophosphate on the sensitivity of sodium efflux from squid axons to external sodium and potassium. *J. Gen. Physiol.* **56**:583–620.

DIPOLO, R. 1973. Calcium efflux from internally dialyzed squid giant axons. *J. Gen. Physiol.* **62**:575–589.

DIPOLO, R. 1974. Effect of ATP on the calcium efflux in dialyzed squid axons. *J. Gen. Physiol.* **64**:503–517.

DIPOLO, R. 1976. The influence of nucleotides on calcium fluxes. *Fed. Proc.* **35**:2579–2582.

DIPOLO, R., J. REQUENA, F. J. BRINLEY, JR., L. J. MULLINS, A. SCARPA, and T. TIFFERT. 1976. Ionized calcium concentrations in squid axons. *J. Gen. Physiol.* **67**:433–467.

HODGKIN, A. L., and R. D. KEYNES. 1957. Movements of labelled calcium in squid giant axons. *J. Physiol. (Lond.)*. **138**:253–281.

MULLINS, L. J. 1976. Steady-state calcium fluxes: membrane versus mitochondrial control of ionized calcium in axoplasm. *Fed. Proc.* **35**:2583–2588.

MULLINS, L. J., and F. J. BRINLEY, JR. 1969. Potassium fluxes in dialyzed squid axons. *J. Gen. Physiol.* **53**:704–740.

MULLINS, L. J., and F. J. BRINLEY, JR. 1975. The sensitivity of calcium efflux from squid axons to changes in membrane potential. *J. Gen. Physiol.* **65**:135–152.

REQUENA, J. 1976. *Act. Cient. Venez.* **25**(1):12–13.

ROJAS, E., and R. E. TAYLOR. 1975. Simultaneous measurements of magnesium, calcium and sodium influxes in perfused squid giant axons under membrane potential control. *J. Physiol. (Lond.)*. **252**:1–27.