Intracellular Calcium Buffering Capacity in Isolated Squid Axons

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ABSTRACT Changes in ionized calcium were studied in axons isolated from living squid by measuring absorbance of the Ca binding dye Arsenazo III using multiwavelength differential absorption spectroscopy. Absorption changes measured *in situ* were calibrated in vitro with media of ionic composition similar to axoplasm containing CaEGTA buffers. Calcium loads of 50–2,500 μmol/kg axoplasm were induced by microinjection, by stimulation in 112 mM Ca seawater, or by soaking in choline saline with 1–10 mM Ca. Over this range of calcium loading of intact axoplasm, the ionized calcium in the axoplasm rose about 0.6 nM/μM load. Similar loading in axons pretreated with carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) to inhibit the mitochondrial proton gradient increased the ionized calcium by 5–7% of the imposed load, i.e. 93–95% of the calcium load was buffered by a process insensitive to FCCP. This FCCP-insensitive buffer system was not saturated by the largest calcium loads imposed, indicating a capacity of at least several millimolar. Treatment of previously loaded axons with FCCP or apyrase plus cyanide produced rises in ionized calcium which could be correlated with the extent of the load. Analysis of results indicated that, whereas only 6% of the endogenous calcium in fresh axons is stored in the FCCP-sensitive (presumably mitochondrial) buffer system, about 30% of an imposed exogenous load in the range of 50–2,500 μM is taken up by this system.

INTRODUCTION

Although the calcium-accumulating capacity of the mitochondrion is legendary, the homeostatic role of this organelle in situations involving physiological concentrations of free calcium has never been clearly delineated. This role deserves study, especially in view of the recent demonstrations that mitochondrial calcium uptake is a strong function of the free magnesium concentration. When physiological concentrations of free magnesium are present (i.e. 1–3 mM) the calcium concentration for half-maximal rate of uptake is in the range of tens of micromolar (8*a*). In view of the fact that the free calcium concentration in many cells is of the order of tens of nanomolar, i.e. about 1,000 times lower than the concentration for half-maximal uptake, it is possible that the rate of calcium uptake by mitochondria might not be sufficiently rapid to buffer physiological perturbations of ionized calcium. In addition to the mitochondria there are, at

least in squid axons, other organelles (22, 15, 14) as well as soluble entities (1, 3) which can sequester calcium.

Prior studies of the intracellular calcium binding moieties have utilized isolated material. This study represents an initial attempt to define in isolated giant axons of squid the function of these entities *in situ* by using differential absorbance changes in the metallochromic dye, Arsenazo III, as a nondestructive technique for rapid continuous monitoring of the ionized calcium. Although this experimental strategy may permit more definite statements about the physiological function of mitochondria, the use of isolated excitable axons severely limits the extent to which the composition of the axoplasm can be altered. Furthermore, a study of buffering systems in a marine invertebrate in which the ionic strength and free magnesium concentrations are higher than in mammalian preparations may lead to conclusions which are not of general validity. These limitations of the present study should be kept clearly in mind.

MATERIALS AND METHODS

Material

Experiments were performed during May and June, 1976, with live specimens of *Loligo pealei* obtained from the Marine Biological Laboratory, Woods Hole, Mass. Axons were dissected with the mantle immersed in flowing, chilled, natural seawater, and subsequently cleaned in 3 mM calcium seawater. Most experiments were performed with freshly isolated axons. A few experiments were done with axons which had been stored for several hours in 3 mM calcium artificial seawater at 10°C.

Apparatus

MOUNTING CHAMBER AND MICROINJECTOR. The chamber used to hold the fibers was similar to that described in earlier publications (12). The microinjector used for introducing materials inside the mounted squid axon was similar to that described earlier (5) except that it was modified so that the injector could be introduced into the axon by motion in a horizontal rather than a vertical direction. The injection syringe was either a 0.5 μl Hamilton syringe (delivering .083 μl/cm of travel) or a 1.0 μl Hamilton syringe (delivering 0.18 μl/cm of travel).

APPARATUS FOR OPTICAL MEASUREMENTS. The basic principles involved in using metallochromic dyes and differential absorption spectroscopy to measure metal ion concentrations have been reviewed by Scarpa (26). Their application specifically to measurements in large invertebrate muscle and nerve fibers have been described in prior publications (6, 12, 7). Only significant improvements in technique are described here.

The apparatus for measuring absorbance changes in squid axons is illustrated schematically in Fig. 1. Light was directed through the axon mounted in the chamber by means of glass light pipes. The entrance light pipe had dimensions of 12 mm × 0.3 mm and was adjusted so that the light passed through the longitudinal axis of the axon. The collecting light pipe had dimensions of 12 mm × 3 mm and was also symmetrically positioned with respect to the longitudinal axis of the axon. Although the light pipes were fixed in the vertical plane, they could be adjusted horizontally so as to be placed in close apposition to the fiber in order to collect more scattered light. With these improvements the overall sensitivity of the instrument was approximately two- to threefold greater than that of the system described earlier.

The microspectrophotometer used in these experiments was similar to one used previously (6), except that an eight-channel rotating wheel (rotation speed 3,000 rpm) was

used which permitted measurements of differential absorbance changes at three wavelength pairs in addition to the isosbestic point. This procedure permitted the measurement of absorbance changes in wavelength pairs unaffected by changes in internal calcium and afforded a convenient check on the ability of the technique to discriminate against absorbance changes unrelated to ionized calcium. The wavelengths used for calcium were either 685–675 or 685–660 nm because these pairs are relatively insensitive to magnesium. The absorbance was displayed on a recorder at a usual gain of 0.0007 ΔA/cm.

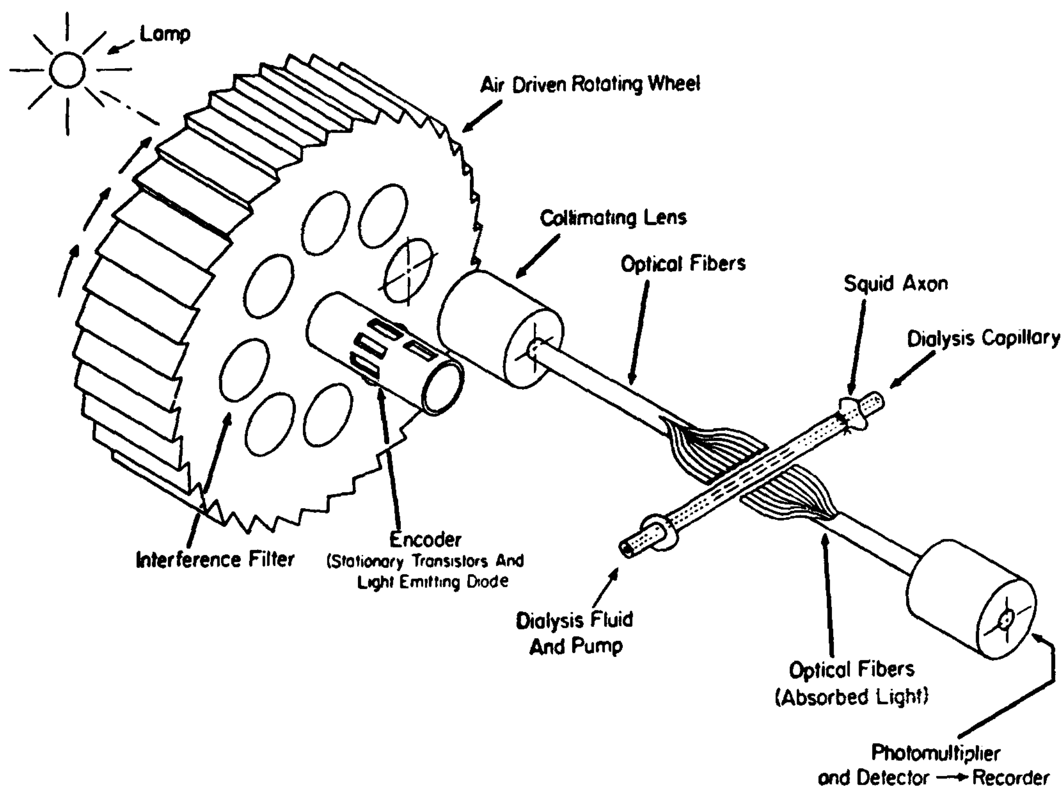

FIGURE 1. Schematic diagram of the pulsed multiwavelength microspectrophotometer used in these experiments. Squid axon is positioned in the lightpath which is formed by an array of glass optical fibers. Light from a quartz iodide lamp passes through a series of monochromatic interference filters mounted on a wheel driven at approximately 3,000 rpm by a jet of compressed air. It then passes through the preparation and is detected by a photomultiplier. The signal from the photomultiplier is converted to a series of absorbances at eight wavelengths. The differential absorbance between selected pairs of wavelengths is displayed on recorder.

CALIBRATION OF ABSORBANCE CHANGES. Some theoretical and practical considerations in the calibration of Arsenazo III were considered in an earlier publication (12). In the present experiments, calibration of the absorbance changes produced in an axon, in terms of changes in ionized calcium, was obtained as follows. The axon was replaced by a quartz glass capillary with an inside diameter of 600 μm. This diameter was selected to be reasonably close to the diameter of the axons used in this study (450–700 μm). Calibrating solutions were made up with samples of the same dye as was microinjected into the axons. The concentration of the dye in the calibrating solution was adjusted to be close to that

obtained in the axon. When the dye solutions were to be calibrated for ionized calcium in the 50–100-nM range, aliquots of stock calcium EGTA buffer solutions with these concentrations were added directly to the calibrating solutions. When the dye was to be calibrated for ionized calcium concentration in the 10–50-μM range, samples of 100 mM calcium chloride standard were added directly to the calibrating solutions. These calibrating solutions were then flushed through the quartz capillary and the absorbance changes were measured. The absorbance changes were converted to changes in ionized calcium appropriate for a particular axon, on the assumption that the absorbance change was linear with respect to both concentration and length of light path.

Linearity of the Calibration

The calibration procedure described above is equivalent to calculating ionized calcium from the relation:

$$Ca_i = \frac{(Ca\ A_3)}{A_3} \cdot K_D,$$

where K_D is the dissociation constant for calcium-arsenazo III. Since K_D is about 30–50 μM under the conditions of these experiments (μ = 0.4, pH = 7.3), and total concentration of arsenazo III ranged from 0.5 mM to 2 mM, the amount of dye complexed to calcium was never more than a few percent of the total for ionized calcium between 0 and 1 μM. For higher ionized calciums, the amount of arsenazo complexed ranged from 10% to 20%. Although the binding introduces some nonlinearity it was not considered serious in view of the other uncertainties in the calibration.

Buffering Capacity of Arsenazo III

Because rather large concentrations of arsenazo III were used in these experiments, it is necessary to consider the extent to which the injected arsenazo can buffer the internal ionized calcium. The ratio between free calcium and that bound to arsenazo III is given by K_D/A_3. Given the dissociation constant for arsenazo and the usual final concentration of the dye in the axoplasm, the concentration of calcium bound to the dye is around 30 times greater than the free calcium.

If the ionized calcium is in the physiological range, i.e. 20–50 nM, then the concentration of calcium complexed to arsenazo is 600–1,500 nM, which presumably would be extracted from releasable internal stores, if calcium-free arsenazo were added to the axoplasm. Actually, the arsenazo used had between 0.5% and 1% mol fraction calcium contamination, so that several micromolar calcium was usually being added to the axoplasm rather than removed from it. Presumably, this calcium was taken up by the internal buffering capacity of the axon.

The buffering capacity of arsenazo is probably not important in experiments in unpoisoned axons since it will be shown that in those cases the ratio of free to bound calcium is ca.1:2,000, i.e. the dye buffers weakly compared to intact axoplasm. However, in certain experiments in which axons were poisoned with FCCP, the residual cellular buffering capacity appeared comparable to that of the dye itself. This complication is considered further in the discussion of these experiments.

Calculation of Concentration of Arsenazo III in the Axoplasm

The concentration of arsenazo *in situ* was not measured directly but was calculated from the known concentration of arsenazo in the fluid microinjected into the axon and the dimensions of the fiber. To the extent that arsenazo is bound *in situ* to moieties other than calcium, in vitro calibration will be in error.

Probably a significant amount of arsenazo is bound to magnesium. Given a K_D for Mg A_3 of about 6 mM (12), and an ionized magnesium of about 3 mM (6, 11), it can be

calculated that about one-fourth of the arsenazo in axoplasm is bound to magnesium. Since the calibration solutions did not contain magnesium, the calibration will be in error by this factor, which would have the effect of raising the calculated ionized calcium concentrations by this factor.

The concentration of arsenazo III present in the axoplasm was calculated on the assumption that the dye was pure. It is now known that at the time these experiments were done, only about one-half of commercially available dye was actually arsenazo III. Since calibrations and dissociation constant measurements for both calcium and magnesium were done on the same batch of dye (from which calcium had been removed), these circumstances should not affect the calibration, unless in vitro binding of the impurities and of arsenazo varied greatly.

Tests to evaluate the extent of arsenazo III binding to cell or cell fractions were performed as follows. Different concentrations of dye were added to high concentrations of suspensions of ascites tumor cells, erythrocytes, rat liver mitochondria, rat liver microsomes, bovine chromaffin vesicles, and rabbit soleus sarcoplasmic reticulum. After mixing, the particulate matter was separated from the supernate by centrifugation, and the concentration of arsenazo III in the supernate was measured at the isosbestic point (arsenazo vs. Ca-arsenazo). The concentration in the supernate was identical to that in the original solution before addition of particulate matter, indicating lack of binding to the latter.

Solutions and Reagents

The external solutions used in these experiments had the composition as indicated in Table I of Requena et al. (23). Solution CBT, which was used to calibrate the absorbance changes, had an ionic strength and pH chosen to approximate that of axoplasm (10). Arsenazo III (1,8-dihydroxynaphthalene-3,6-disulfonic acid-2,7-*bis*{(azo-2)-phenylarsonic acid}) was obtained from Sigma Chemical Co., St. Louis, Mo. The commercial material was purified as described by DiPolo et al. (12). The calcium contamination after purification was approximately 0.01 mol fraction. Carbonyl cyanide, *p*-trifluoromethoxyphenylhydrazone (FCCP) was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml. 2–5 μl aliquots were applied to the surface of the axon with a 10μl Hamilton syringe. Apyrase, obtained from Sigma Chemical Co., was found to be significantly contaminated with calcium. This was removed by twice passing a solution of the enzyme through a Chelex 100 column. Commercial lots of potassium isethionate and aspartate also proved to have significant calcium contamination, which was removed by passing stock solutions through a Chelex 100 column. Subsequent analyses of samples by atomic absorption spectrophotometry indicated the residual calcium contamination was 0.001% mol fraction.

Isotope Experiments

Radioactive ^{45}Ca was obtained from New England Nuclear (Boston, Mass.) as the chloride and dried in a crucible. Appropriate amounts of seawater were added to give the desired specific activity. Radioactive samples were counted in a liquid scintillation counter (Beckman LS-230, Beckman Instruments, Fullerton, Calif.) with a commercially available counting fluid (Hydromix, Yorktown Research Inc., S. Hackensack, N. J.).

RESULTS

Experiments on Isolated Axoplasm

Multiple microinjection experiments were all conducted on samples of extruded axoplasm sucked into a 600-μm ID quartz capillary. Placing the glass microinjec-

tion capillary in the lightpath produced a substantial change in absorbance roughly equivalent to the absorbance change produced by a 20-nM change in ionized calcium. Fortunately, it was found that backing the injection capillary out of the lightpath without injecting calcium returned the absorbance reading to the base-line levels, so that correction of records for this artifact was not necessary.

An example of the absorbance change produced by injecting a sufficient amount of calcium chloride into isolated axoplasm to produce a final concentration of 150 μM (assuming no buffering) is shown in Fig. 2. The ordinate represents average ionized calcium concentration in the axoplasm.

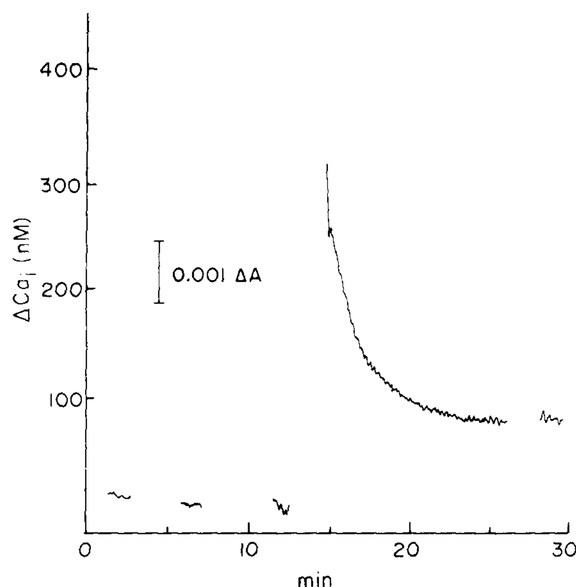

FIGURE 2. In vitro experiment with axoplasm inside a 600-μm quartz capillary. Sufficient calcium chloride to produce final concentration in the axoplasm of 150 μM was microinjected at time 15 min. A new steady-state level of absorbance was obtained after about 10 min, corresponding to an increase in ionized calcium of approximately 80 nM. The initial base line was interrupted by motion artifacts due to moving the injection capillary into and out of the light path.

There are three points of interest with respect to the data.

(*a*) A large fraction of the buffering must occur very rapidly. The basis for this statement is the finding that the earliest absorbance measurement that can be made after the injection is equivalent to an ionized calcium of only a few hundred nanomolar. The injection fluid contained about 1 mM Ca. This concentration of calcium, in the initial line injection, would completely saturate the dye molecules in the vicinity. Therefore the initial absorbance change does not represent a mean concentration and cannot be used to indicate the initial amount of unbuffered calcium present. However, control experiments in which small amounts of calcium were injected into the calibrating solution (CTT) showed clearly that initial absorbances equivalent to 50 μM ionized calcium could be observed.

We conclude that virtually all of an injected load was buffered too rapidly for us to resolve it on the time scale of these experiments.

(*b*) Despite the fact that there is a large amount of extremely rapid initial binding of calcium to intracellular material, final diffusional equilibrium of the ionized calcium is relatively slow and is reached with a half-time of 5–10 min. This half-time agrees well with a half-time of 7–10 min found by Blaustein and Hodgkin (4) for the diffusion of ^{45}Ca to the periphery of an axon after axial injection of calcium chloride for a diffusion path of 400 μm in the axon, compared to 300 μm for the in vitro experiments.

(*c*) The final steady-state level of ionized calcium is about 0.1% of the steady-state concentration which should have been obtained on the basis of the amount injected.

Fig. 3 shows a titration curve obtained by repeated injections of calcium into

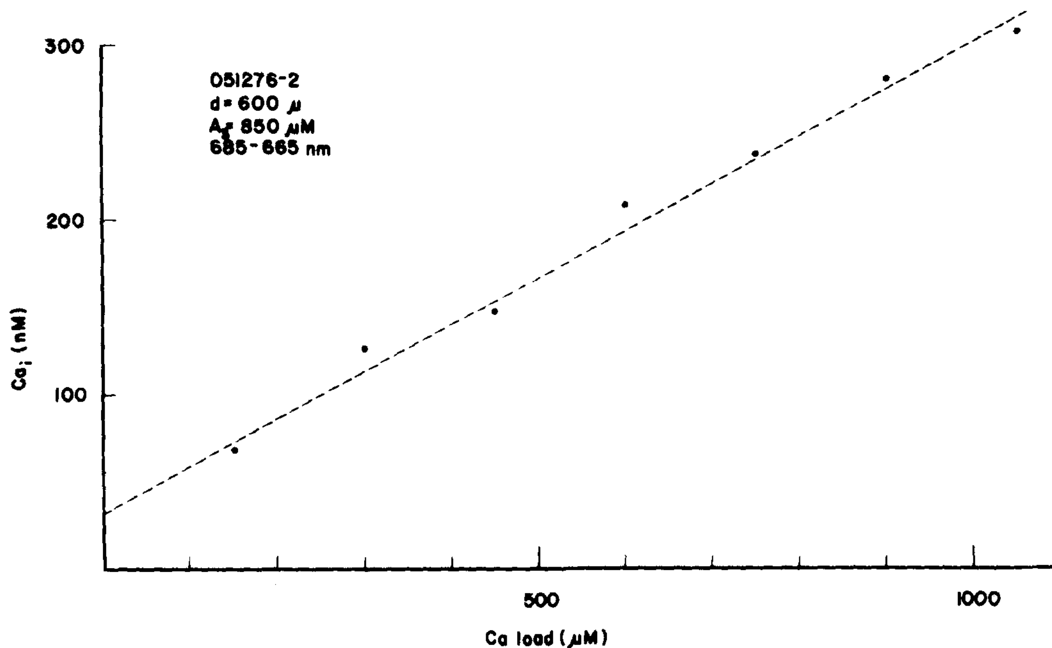

FIGURE 3. In vitro titration curve produced by repeated injections of calcium chloride into extruded axoplasm drawn up in 600-μm glass capillary. Abscissa, Calculated calcium load assuming uniform distribution of injected calcium. Ordinate, Final steady-state increment in ionized calcium, obtained from in vitro calibration curves relating absorbance and free calcium.

samples of axoplasm contained in a quartz capillary. The ordinate is the measured ionized calcium and the abscissa shows the calculated calcium load assuming uniform diffusion of injected calcium. The data show clearly that the ionized calcium increases linearly with calculated total loads approaching 1 mM (which is about 20 times the endogenous calcium content of squid axons) without indication of any evidence of saturation of the calcium buffer system in extruded axoplasm. The rise in ionized calcium per injection is 0.3–1 nM/μM calculated load.

It has previously been demonstrated (2, 4, 12), that treatment of isolated axons with 2 mM cyanide produces an increase in ionized calcium. This increase presumably results from the release into axoplasm of internal stores of calcium which had been held in unionized form. A few experiments were done to confirm that such release could occur in isolated axoplasm which had previously bound a known amount of calcium. The experiment illustrated in Fig. 4 is one

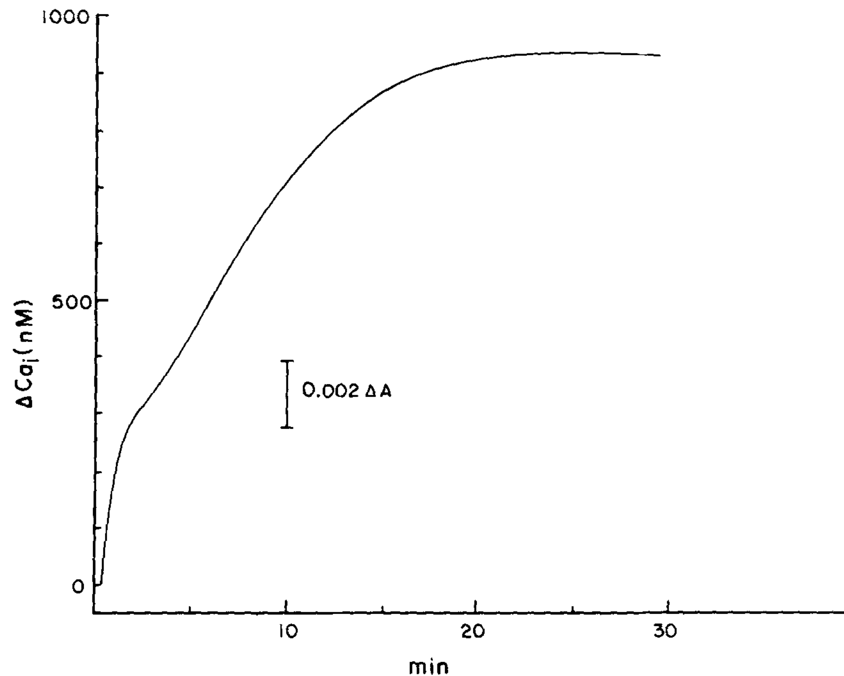

FIGURE 4. In vitro experiment on axoplasm drawn up into 600 μm ID quartz capillary tube. Axoplasm had been preinjected with apyrase and with sufficient calcium to give a final concentration of 320 μM. At time 0, bolus of buffered potassium cyanide, sufficient to give a final concentration in the axoplasm of 2 mM, was microinjected. Note that although this sample of axoplasm had received an exogenous calcium load of about 320 μM calcium, the ionized calcium rose only about 1 μM after cyanide treatment. Onset of cyanide effect is immediate because of prior injection of apyrase.

such experiment. The axoplasm had previously been injected with the enzyme apyrase to reduce the axoplasmic level of ATP, although of course it should not affect the utilization of substrate by the mitochondria. It can be seen that immediately upon the injection of a concentration of cyanide calculated to give a final concentration of 2 mM throughout the axoplasm, the ionized calcium began abruptly to rise. The final level of calcium was reached in about 20 min and amounted to about 1 μM of ionized calcium.

Although experiments on isolated axoplasm are useful to demonstrate the marked buffering capacity of axoplasm in the absence of any possible membrane contribution, there is doubt as to the physiological condition of the axoplasm. Axoplasm once placed inside the capillary cannot be further oxygenated. Even

though precautions were taken to keep the tubes of axoplasm cold before use, and to work rapidly once the tubes had been mounted, the experiments took at least 20–30 min to complete. Calculations based on the rate of oxygen consumption for isolated squid axons (9) indicate that axoplasm saturated with room air at 15°C would be essentially depleted of oxygen within 10 min.

Another difficulty with microinjection is the effect that high concentrations of calcium in the injection fluid might have upon mitochondria or other intracellular organelles in or immediately adjacent to the injection path. Because of dilution of the injection fluid it is necessary to inject about 0.5–1.0 mM calcium chloride in order to achieve an increment of the order of 50–100 μM in the axoplasm. Since axoplasm liquifies and disperses in millimolar calcium chloride solutions, it is reasonable to suppose that mitochondria or other organelles in the immediate vicinity of the injected pathway might be badly damaged simply by the high concentrations of calcium chloride in the injection fluid, and their subsequent behavior would not reflect the buffering capacity of intact axoplasm. For these reasons other methods of loading with known amounts of calcium were used with intact axons as described in the following sections.

Methods of Loading Axons with Calcium

STIMULATION. Hodgkin and Keynes (17), first showed that excitation of squid axons was associated with a net inward calcium flux. This effect of stimulation upon net calcium entry has been exploited in the present study as a convenient means for increasing, in a predictable manner, the calcium content of axoplasm. In order to verify that our experimental conditions actually produced a gain of calcium, cleaned axons were stimulated for varying periods of time at 100 impulses/s in a solution of 112 mM calcium seawater containing ^{45}Ca. Stimulation was begun immediately after the fibers had been placed in the solution. After various loading periods (10–60 min) the fibers were removed and washed for 5–12 min in 3 mM Ca artificial seawater and the axoplasm from the central portion of the fibers was extruded onto a plastic tape. During the process of extrusion, great care was taken that the sample of axoplasm was not contaminated with extracellular fluid. The axoplasm was then dispersed in a solution of Hydromix for counting in a liquid scintillation counter. Influx was calculated from the counts contained in axoplasm and specific activity of soak solution. Since the extra efflux with stimulation is very small, the net gain is virtually equal to the unidirectional flux.

The results are shown in Fig. 5 which represents the net uptake of calcium during stimulation and therefore includes both the resting influx in 112 mM calcium seawater and the extra increment due to stimulation. The slope of the line represents a net influx equivalent to 14 pmol/cm^{2}s for 500 μm axons. This result agrees rather well with the earlier data of Hodgkin and Keynes (17) who found a resting influx of 0.5 pmol/cm^{2}s in 112 mM calcium plus an extra influx due to stimulation (100 impulses/s) at a temperature of 16°C (interpolating from their data at 22°C and 8°C) of about 11 pmol/cm^{2}s. The net entry in their experiments was therefore about 11.5 pmol/cm^{2}s for stimulation at 100 impulses/s.

The data shown in Fig. 5 also indicate that the uptake of calcium at constant frequency of stimulation is linear with time for periods of at least 60 min,

corresponding to a total of 360,000 impulses. This linearity of uptake is important for the interpretation of our results because in many of the experiments the loading of the axon by stimulation was continued for 30–50 min in order to put large calcium loads in the axoplasm.

The actual increase in calcium content expressed as micromoles per kilogram of axoplasm, for any given axon will of course depend upon the diameter. However, as a convenient datum for calculation, the rounded figure of 50 μmol/kg axoplasm per minute of stimulation, appropriate to a fiber of 500 μm, has been used as an average value since most axons used in this study had diameters reasonably close to that figure.

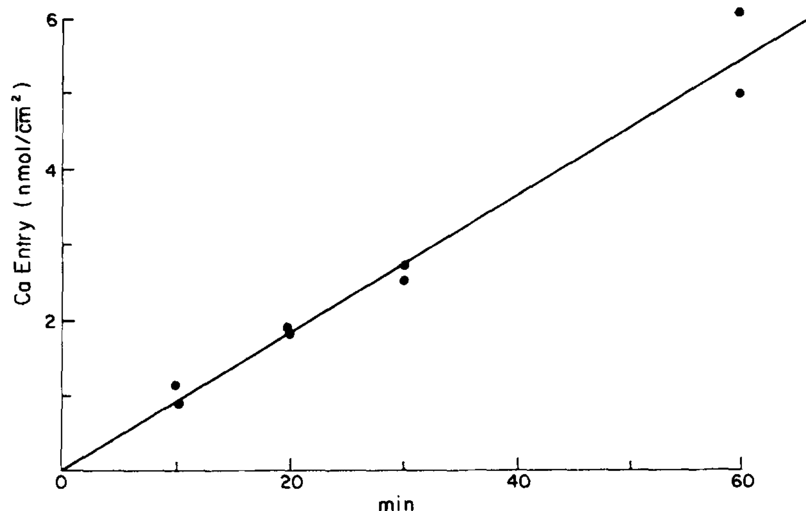

FIGURE 5. Effect of stimulation at 100 impulses/s on calcium entry in squid axons. The calcium load (ordinate) is plotted as nanomoles of calcium which enter per 1 cm^2 of membrane area during the period of stimulation (abscissa). Axons in 112 mM Ca seawater. Net entry = 0.014 pmol/cm^2·imp.

SOAKING IN SODIUM-FREE SOLUTIONS Since soaking isolated squid fibers in sodium-free solutions increases influx (2) and decreases outflux (4), such a procedure should increase the internal calcium content.

The preceding paper (23) provided analytical evidence of this increase, which for a 500-μm axon isolated from *L. pealei* amounts to approximately 40 μmol/kg of axoplasm/min during immersion in a sodium-free saline containing 10 mM calcium. Although we have no direct experimental evidence that the net uptake is proportional to the extracellular concentration, we have assumed it to be so, and have adopted the figure of 4 μmol/kg axoplasm/min/mM extracellular calcium as an average figure for the increase in calcium content of axons bathed in sodium-free solutions.

Release of Endogenous Calcium

Requena et al. (23), using aequorin to monitor ionized calcium, indicated that fresh axons with a Ca content of 50 μM/kg axoplasm release a small but

measurable amount of calcium during prolonged exposure to cyanide. The data indicated that cyanide treatment may triple the ionized calcium concentration (i.e. raise it from ~30 to 90 nM). Other experiments reported in that paper indicate that the addition of FCCP to the bathing media produced a prompt rise in ionized calcium of about the same amount. Since FCCP has been demonstrated to be an uncoupler of oxidative phosphorylation in the mitochondria of a variety of mammalian species, the implication is that the calcium was released from the mitochondria. Similar experiments are reported in this paper.

Fig. 6 shows the effect upon ionized calcium of the application of 3 μl of FCCP

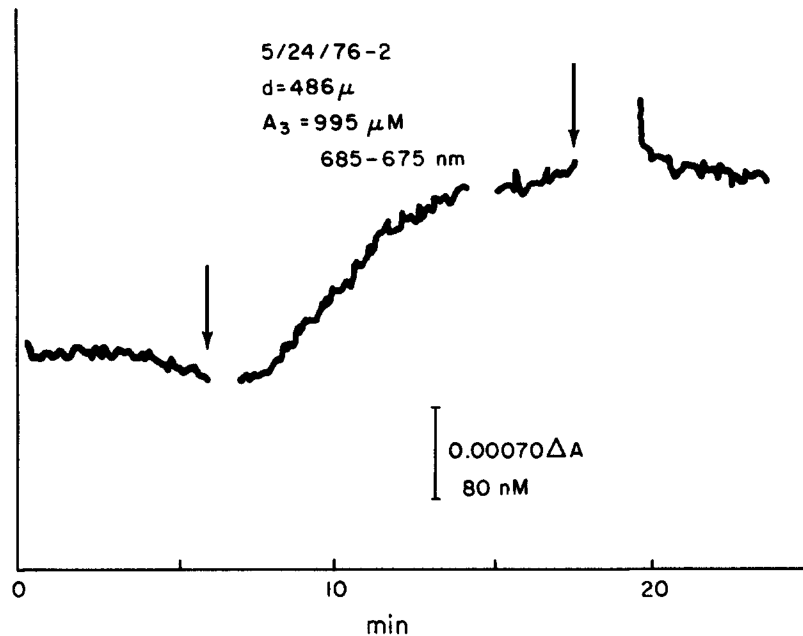

FIGURE 6. Effect of FCCP on release of endogenous calcium from fresh axon maintained in 3 mM calcium-ASW until shortly before experiment, when it was placed in 0 calcium ASW. First application of FCCP produced prompt, maximal change in absorbance corresponding to 120 nM increase in free calcium. Vertical arrows indicate application of 3–5 μl of FCCP (2 mg/ml) in 100% DMSO to surface of axon.

solution (FCCP 2 mg/ml 100% DMSO) to the 1-cm length of axon contained in the lightpath. The figure shows that one application at the first arrow produced a rise in ionized calcium, which in this axon amounted to about 120 nM. The first application was evidently maximal since another addition of FCCP produced no further increase in ionized calcium. The effect typically took about 5 min to become maximal. However this slow release cannot necessarily be related to slowness in the onset of FCCP action. Because of the poor water solubility of FCCP it was necessary to put it in a solubilizing vehicle (DMSO). Some time was required for the DMSO to pass from the surface of the solution, where it was injected with a small syringe, to the surface of the axon and to diffuse, carrying FCCP with it, through the substance of the axoplasm. The effect cannot be

ascribed to the DMSO in which FCCP was dissolved, because in control experiments, application of equal or larger volumes of DMSO produced no absorbance change.

The results from this and five other freshly isolated axons stored in 3 mM Ca are listed in Table I. The average increment in ionized calcium occasioned by maximally effective concentrations of FCCP is approximately 140 nM. Since fresh axons contain about 30 nM Ca_i, the ionized calcium must have risen from 30 to 170 nM, approximately a sixfold increase which is reasonably close to the trebling of ionized calcium seen in FCCP-treated axons with the aequorin method.

TABLE I

RELEASE OF ENDOGENOUS Ca FROM SQUID MITOCHONDRIA

Axon	Diameter	A_3	ΔCa_i
	μm	*mM*	*nM*
5/19/76-3	500	0.8	116
5/20/76-1	470	0.8	150
5/21/76-1	470	1.0	275
5/24/76-2	486	1.0	117
5/25/76-1	458	1.12	160
5/25/76-2	492	1.0	42

The increment in ionized calcium seen in these experiments cannot be directly related to the presumed calcium release by the mitochondria because, as will be shown in subsequent sections, most of a calcium load, however imposed upon the axoplasm, is taken up by an FCCP-insensitive buffer system. The actual release of calcium from the mitochondria is therefore greater than the rise in ionized calcium would indicate. This point is considered in detail in the Discussion.

Axoplasmic Buffering of Exogenous Calcium

It has been shown in a previous section that axoplasm in vitro can buffer virtually all of an imposed calcium load. In this section we present data indicating that buffering also occurs *in situ,* and in addition describe the extent to which a buffered load can subsequently be released by interference with metabolic processes inside the cell. The results of this section provide the basis for a division, explained in the Discussion, of the buffering capacity of axoplasm into metabolically labile and metabolically insensitive components.

Fig. 7 shows an experiment very similar to that of Fig. 6 except that in this case a fresh axon was exposed for 12 min to saline containing 1 mM calcium 0 sodium. During this period the axon gained approximately 4 μmol/kg axoplasm per minute of calcium for a total calcium load of 48 μM. Although not well shown on the scale of this figure, the ionized calcium rose approximately 50 nM during this time. Thus, as in the case of isolated axoplasm, nearly all of the calcium load was buffered. Evidently, some of this calcium was held in a metabolically labile form, because when a maximally effective concentration of FCCP was added (vertical arrow) the ionized calcium rose abruptly and reached

a steady level approximately 850 nM greater than before FCCP. The second vertical arrow represents a second addition of FCCP which was without effect. Since the calculated load was 48 μM, the result of this experiment indicates that even in the presence of maximally effective concentrations of FCCP, which should completely block the mitochondrial proton gradient, a very substantial amount of calcium remains buffered.

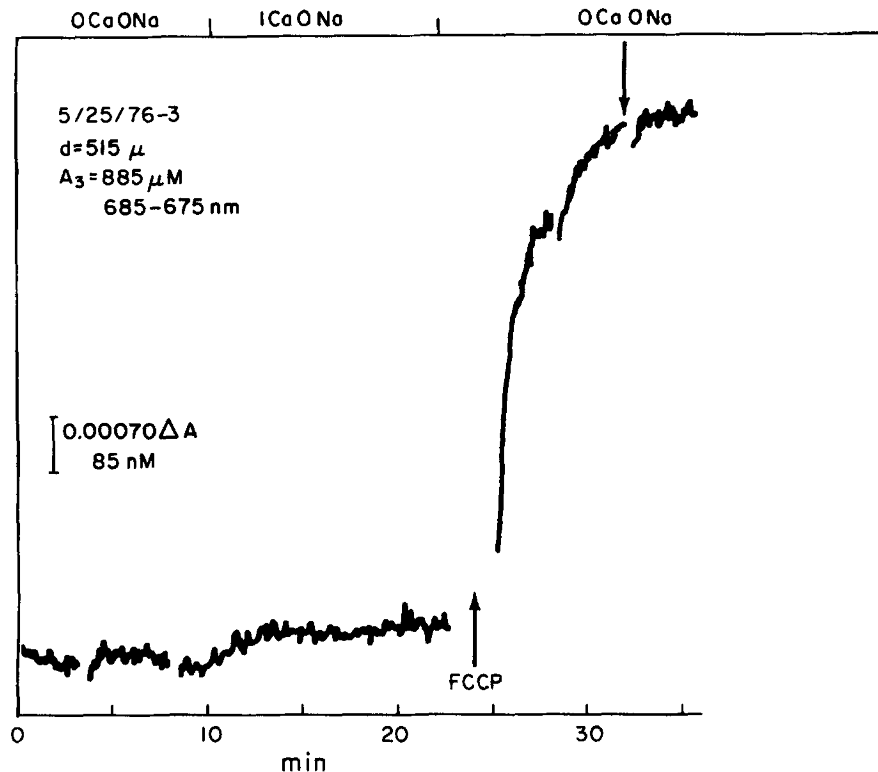

FIGURE 7. Experiment similar to that of Fig. 6, but axon was first loaded for 12 min in 1 Ca, 0 Na solution. Axon returned to 0 Ca 2 min before application of FCCP (vertical arrows). Absorbance change equivalent to 750 nM increase in free calcium. Soak in 1 Ca, 0 Na seawater produced calculated increase in calcium content of 48 μM.

Fig. 8 shows a somewhat different protocol but illustrates the same point. In this experiment a fresh unloaded axon was exposed to FCCP and the ionized calcium in the axoplasm increased about 100 nM. A second addition of FCCP was made a few minutes later, and shortly thereafter the fiber was exposed to saline containing 1 mM calcium, 0 sodium. In this solution the fiber should gain roughly 4 μmol/kg axoplasm per minute, which is the same load imposed on the fiber shown in Fig. 7. However, in this case the mitochondria are still inactivated by FCCP and one can see that the rise in ionized calcium is very much greater than that of an axon with functional mitochondria.

Inasmuch as the effect of FCCP is transient, the mitochondria ultimately regain their ability to reaccumulate calcium and the concentration of ionized calcium returns to a much lower level, although the fiber remains in 1 mM calcium 0 sodium and presumably continues to gain calcium at roughly 4 μM/

FIGURE 8. Calcium uptake in axon treated with 3 μl FCCP before exposure to 1 Ca, 0 Na. Axon initially immersed in 0 Ca, 0 Na to stabilize internal calcium content. First application produced slight rise in ionized calcium which was maximal because second application produced no further increase. Axon was then exposed to 1 Ca, 0 Na ASW while the FCCP effect was still present. This produced a marked rise in ionized calcium which was not sustained, the ionized calcium returning to the initial FCCP treatment level with a time constant of approximately 20 min. Substantial increase in calcium content of the fiber during exposure to 1 Ca, 0 Na saline is indicated by final FCCP treatment which released sufficient calcium to raise the ionized level to about 2.5 μM.

min. The steady-state level of ionized calcium in this solution after reversal of FCCP effect is about 100 nM greater than in the unloaded condition. Although the re-establishment of the FCCP-sensitive buffer system has led to a nearly normal ionized calcium, it cannot be inferred that all of the reaccumulated calcium, as well as the calcium continuing to enter the fiber during exposure to 1 mM Ca, 0 Na, was being held in a mitochondrial buffer system. The reason for

this statement can be seen from the final procedure in the experiment which was another exposure of the fiber to FCCP producing a rise in ionized calcium of 2.5 μM. Even though this rise in ionized calcium is far larger than is seen in fresh axons, it still accounts for only a very small fraction of the total calcium load, which by the end of the period of exposure to sodium-free solution was estimated to be 160 μM.

Rapid reversal of the initial FCCP effect was invariably seen in experiments similar to that shown in Fig. 8. We assume this reversal to have the same explanation as has been given for the phenomena in vitro, i.e. binding to proteins (19). Consistent with this explanation is the observation that reversal of the FCCP effect was slower and less complete with repeated additions.

The preceding results cannot be ascribed to some peculiarity of action of FCCP, because quantitatively similar results are seen when fibers which have previously been exposed to exogenous calcium loads are treated appropriately with cyanide-containing solutions as discussed in the following section.

Effect of Metabolic Inhibition by Cyanide upon Ionized Calcium

Fig. 9 shows the effect of cyanide upon ionized calcium in an axon preloaded by soaking for 10 min in 10 Ca, 0 Na seawater (which imposed a calcium load of about 400 μM). The effect of such preloading as indicated by the first elevation in the trace, was a transient increase in ionized calcium of about 125 nM. The ionized calcium returned essentially to the base line within 2 min after return of the fiber to 3 mM Ca artificial seawater.

Except for the loading period, the fiber was kept in a solution that was 0 sodium to minimize outward calcium pumping, and also was 0 calcium to prevent calcium entry during the period in cyanide. CN was applied during the interval indicated by the dotted line. The figure shows that after the usual 2–3-h delay, the ionized calcium rose gradually to about 1.5 μM, indicating that some of the calcium that entered during the loading period was being held in a metabolically labile compartment. As in the case of FCCP-treated axons, the rise in ionized calcium was only a small fraction of that calculated to have entered the fiber during the loading period, indicating that CN-insensitive compartments must hold substantial amounts of calcium.

Fig. 10 shows a similar experiment except that in this case the fiber had been preinjected with apyrase to reduce the axoplasmic ATP to near zero (11). As in the experiment of Fig. 9, a loading period in 10 Ca, Na produces only a small rise in ionized calcium which in this case is only partially reversed after return of the fiber in 0 Ca 0 Na. Since the axoplasmic ATP is near zero, inhibition of respiration by CN produces a prompt increase in ionized calcium rather than the delayed rise observed when respiration is inhibited in an axon with a normal compliment of ATP. At the end of the experiment, after return of the ionized calcium to the precyanide level, the fiber was treated with FCCP which produced a rise in ionized calcium to the same final level as seen in CN.

This result demonstrates the equivalence of CN and FCCP treatment, and also shows that however inhibition of the metabolically labile component of calcium sequestration is accomplished, only a small fraction of it appears as free calcium in the axoplasm.

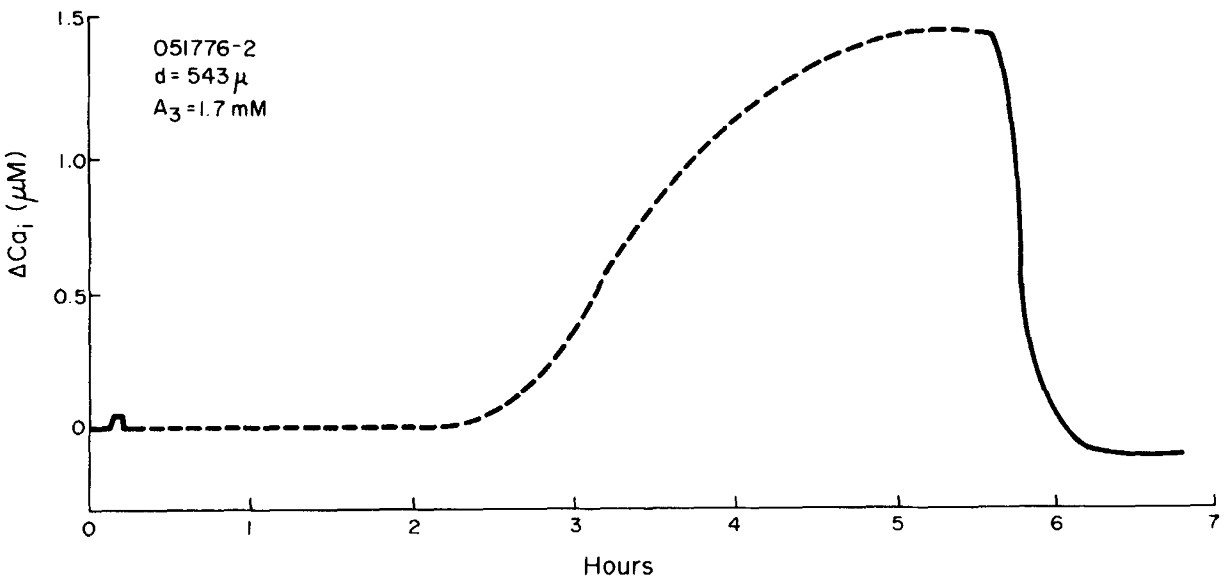

FIGURE 9. Effect of cyanide upon calcium release from squid axon. Fiber was initially loaded with calcium by a 10-min soak in 10 Ca, 0 Na solution which produced slight rise in ionized calcium. Ionized calcium returned to base line after return of fiber to 0 Ca, 0 Na solution. Fiber maintained in 0 Ca, 0 Na solution during entire time to stabilize internal calcium content as much as possible. Dotted line indicates period of immersion in 2 mM CN, 0 Ca, 0 Na ASW. Approximately 2 h after cyanide treatment there is a rise in ionized calcium to about 1.5 μM which is immediately reversed upon removal of the cyanide solution. Note that rise in ionized calcium is only a small fraction of the calculated load introduced by initial brief period in 0 Ca, 0 Na seawater. Figure redrawn from recorder tracings.

Buffering Capacity of Heavily Loaded Axons

In several axons intracellular calcium loads of about 2 mM calcium were induced by prolonged stimulation in 112 mM calcium at 100 impulses/s. A typical result is seen in Fig. 11. After an initial rise of a few micromolar, the ionized calcium

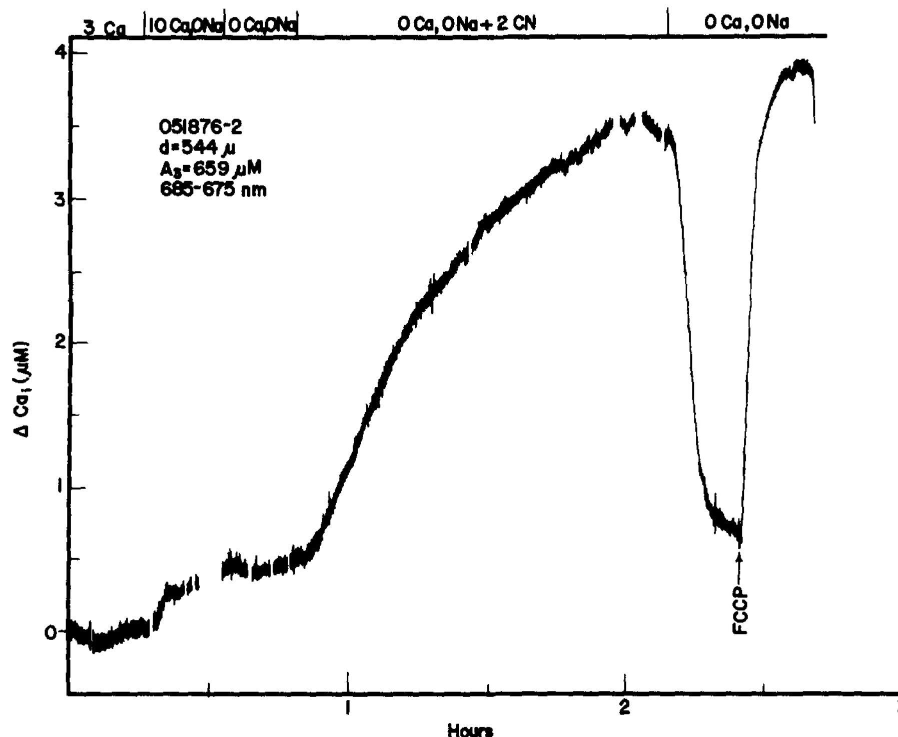

FIGURE 10. Effect of cyanide upon calcium release from an axon preinjected with apyrase to reduce internal ATP. Fiber initially loaded with approximately 720 μM total calcium by an 18-min soak in 10 Ca, 0 Na seawater. Application of cyanide produced immediate rise in internal calcium, indicating that maintenance of intracellular ionized calcium was due largely to mitochondrial respiration. Because of prior apyrase injection which reduced exogenous ATP, when substrate utilization was blocked by cyanide there was no reservoir of ATP to maintain energized calcium sequestration by the mitochondria and free calcium rises. Effect is reversible and ionized calcium returns to precyanide level immediately upon removal of cyanide. Subsequent application of FCCP produces immediate rise in ionized calcium to essentially maximum level obtained in the presence of cyanide.

begins to rise linearly at a rate of about 0.4 nM/μM at an essentially constant rate as the load increases to 2,250 μM during continued stimulation.

Experiments such as these in which the axons are calculated to have been loaded with millimolar concentrations of calcium afforded an opportunity to verify the magnitude of the load by direct analytical measurement of the total calcium in the central segment in the axon. Because of the short length (12 mm)

of the central segment, extrusion of axoplasm was not feasible and the central section was simply cut out (after being washed in Ca-free saline) and analyzed. Results of four analyses are given in Table II where it can be seen that the

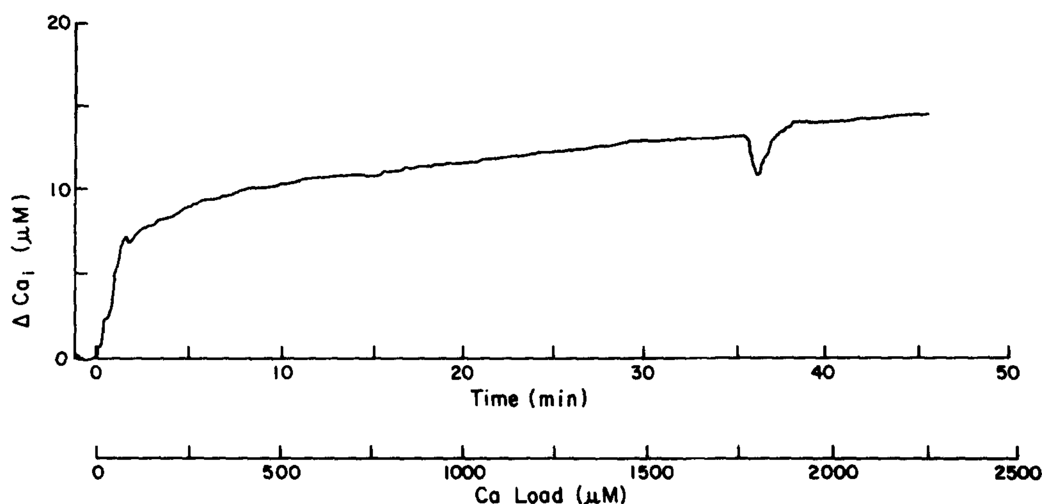

FIGURE 11. No treatment, axon injected with Arsenazo III only. Note that ionized calcium increases immediately after stimulation from the resting of Ca, 50 nM, to 5-10 μM within a space of 2-3 min. Then the concentration of ionized calcium remains virtually constant for about 1 h. The notch in the record resulted from a brief (ca. 30 s) interruption of stimulation.

TABLE II

CALCULATED and MEASURED CALCIUM LOAD IN HEAVILY LOADED AXONS

Axon	Calculated*	Analytical
	mM	
6/3/76-2	3.3	5.0
6/3/76-3	4.2	5.4
6/3/76-4	2.1	2.1
6/4/76	1.0	4.0

*Calculated total load at end of experiment immediately before removal for analysis.

analytical calcium in three of four stimulated axons is very close to or slightly greater than the load calculated from the stimulation (50 μM/min). The fact that the analytical calcium is higher than the calculated load is not surprising since the fibers had been immersed for 30-50 min in 112 mM calcium and were rinsed

in calcium-free solutions for only 10–15 min. It is quite likely that some extracellular calcium remained in the connective tissue in the Schwann cells and this would have been included in the analysis of the central segment which included not only the axon but also the connective tissue on the outside.

Fig. 12 illustrates an experiment in which, at the end of stimulation, the fiber was treated with FCCP. This treatment produced a large increase in ionized calcium (32 μM) indicating that in this axon, as in lightly loaded axons, some of the exogenous load must have been taken up by the FCCP-sensitive system, but that a great deal is sequestered in a compartment unaffected by FCCP.

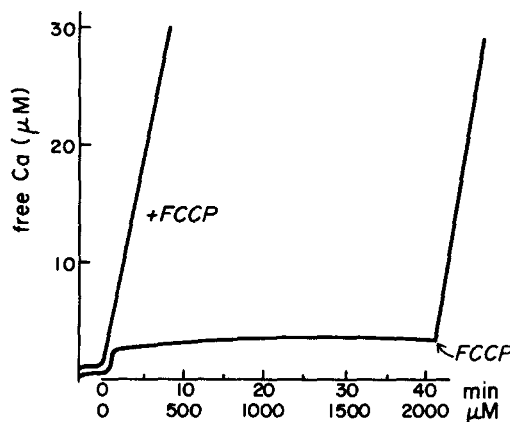

FIGURE 12. Comparison of rise in ionized calcium during stimulation in presence and absence of FCCP; tracings of strip chart records. The abrupt rise in ionized calcium when the intact axon is exposed to FCCP proves that calcium actually entered during the period of stimulation but was taken up nearly completely by internal buffers, presumably mitochondria.

Fig. 12 also shows another experiment in which the fiber was treated with FCCP before stimulation in 112 mM calcium. In this case the FCCP-sensitive buffer system was inactivated before stimulation so that the calcium load was partitioned only between ionized calcium and calcium bound to the FCCP-insensitive buffer system. The results show that stimulation of the axon resulted in a nearly linear rise of ionized calcium. The slope of the curve indicated that about 6% of the load appeared as ionized calcium, the remainder evidently having been buffered by the FCCP-insensitive system. A similar result is shown in Fig. 13 for another FCCP-treated fiber in which the stimulation was interrupted at intervals. During the initial period of stimulation a linear rise of ionized calcium occurred which was 7% of the injected load.

An important conclusion from experiments of this sort is that the FCCP-insensitive buffer system can take up ~93–94% of an imposed load. This result allows one to calculate the total calcium load released in response to FCCP simply by multiplying the rise in ionized calcium by $1/0.06 = 16.7$. Further consideration of this point will be deferred until the Discussion. Since the buffering capacity of the axon is virtually the same at the end of stimulation as it is after the first 5–10 min, we infer that the buffers are nowhere near saturation even at loads 50 times

the normal calcium content. Tests for saturation were not made because the axons became inexcitable after ca. 60 min of stimulation.

Metabolic Requirements for Buffering

Figs. 14 and 15 provide some information concerning the metabolic requirements of the FCCP-sensitive buffer system. The axon in Fig. 14 was preinjected with apyrase about 30 min before starting stimulation. Comparison of the experiment illustrated in Fig. 14 with that illustrated in Fig. 11 shows that the

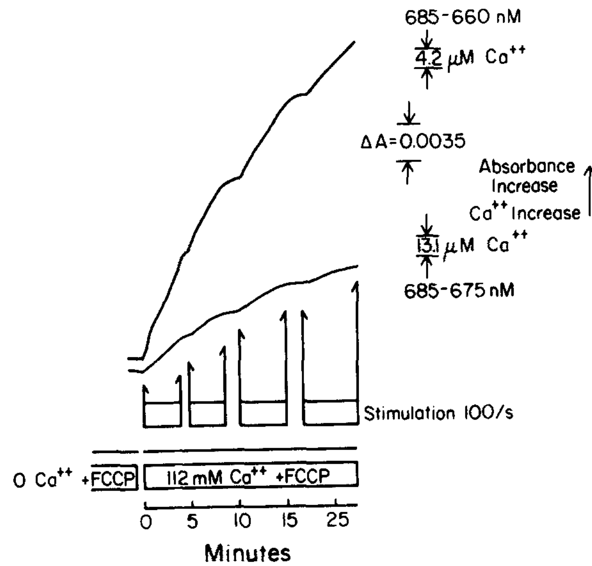

FIGURE 13. Increase in ionized Ca^{++} concentrations in a squid axon electrically stimulated in the presence of high concentration of Ca^{++} and an uncoupler of the oxidative phosphorylation. The axon was microinjected with Arsenazo III, 240 μM, after diffusional equilibration. Where indicated, the bathing solution was changed to seawater containing 112 mM Ca^{++} and the axon was stimulated at a rate of 100 pulses/s. FCCP was superfused over the axon with a motor-driven Hamilton syringe at a rate of 0.2 μl/min. Under those conditions the effect of FCCP was maximal since further additions of FCCP to the axons had no additional effect on ionized Ca^{++} changes (control experiments not shown).

overall buffering capacity of the axon is unaffected by the virtual absence of ATP in the cytosol. The result establishes that endogenous substrate utilization is sufficient to support normal axoplasmic buffering up to calcium loads at least 45 times normal (i.e. 2,250 μM total calcium load).

Fig. 15 illustrates the necessity of respiratory substrate for calcium buffering. In this experiment the fiber was exposed to 2 mM cyanide seawater at time zero when stimulation was begun. This concentration of cyanide is sufficient to block respiration in squid axons immediately (13). However, previous work (8) has established that the initial effect of cyanide on high-energy phosphate compounds in the axoplasm is largely a reduction in phosphoarginine while maintaining the ATP concentration, and presumably ATP to ADP ratio, at near

normal levels for at least 10–20 min. As shown in this experiment, the axoplasmic buffering capacity is impaired from the beginning of loading. By the time the fiber has received a load of about 500 μM, the ionized calcium has risen about 20 μM or several times the rise observed in intact or ATP-depleted axons. This result in conjunction with the apyrase experiments establishes that endogenous substrate is both necessary and sufficient for normal axoplasmic buffering, whereas ATP alone is neither necessary nor sufficient for such buffering.

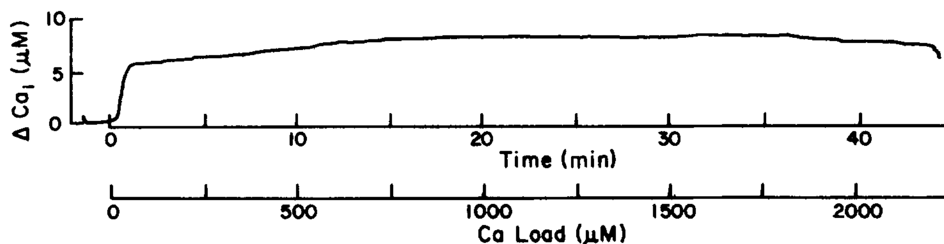

FIGURE 14. In this experiment, the axon was preinjected with apyrase which reduces the ATP axoplasmic concentration from the normal of ca. 4 mM to ~1–2%, i.e. 50–100 μM. Loss of ATP does not affect the ability of the mitochondria to accumulate calcium. The time course of rise of ionized calcium is virtually the same as in the presence of ATP. The experiment implies that substrate alone will energize a normal amount of calcium sequestration by the mitochondria.

This conclusion need not apply to the situation in which the calcium load has been previously imposed upon the axon before cyanide treatment, e.g., Fig. 9 (see also references 2, 12, and 23) where it is clear that cyanide treatment results in calcium release only after a delay of 1 h or more.

Relation of Ionized Calcium to Calcium Content

Some of the experiments already described permitted a correlation between the steady-state ionized calcium concentration and the calcium content of fibers. These and other data are collected in Fig. 16. The relation among the points is reasonably linear over the range of 50 μM (fresh axon) to 1,000 μM with a slope

of about 0.6 nM increase in ionized calcium per micromolar increase in internal calcium content. The data include in vitro experiments, as well as experiments on intact axons in which the load was introduced either by stimulation or by soaks in sodium-free solutions. It also includes two unpublished experiments by

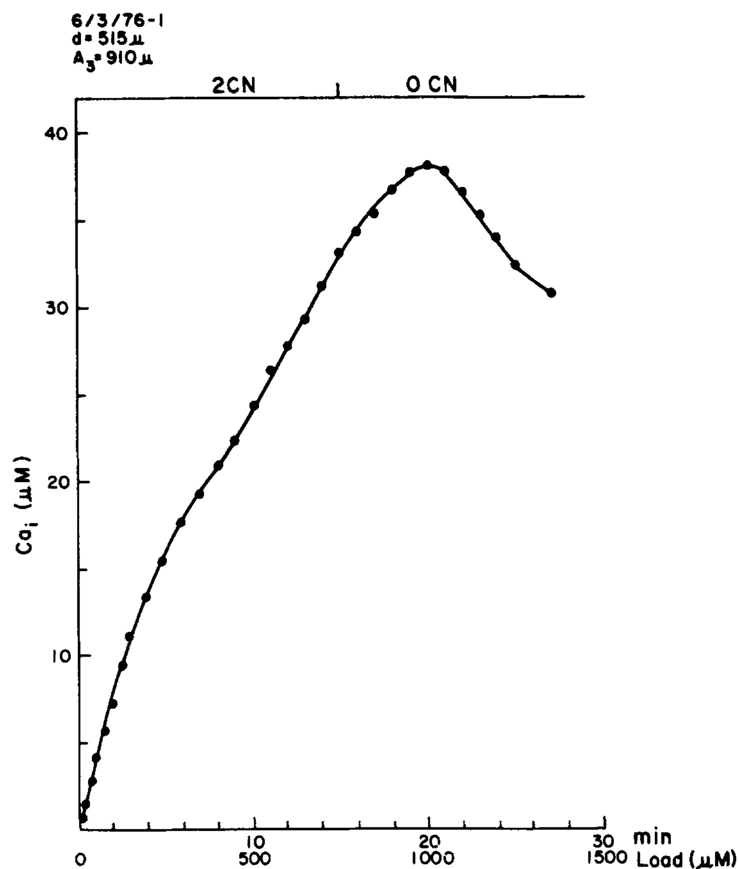

FIGURE 15. In this experiment the axon was exposed to 2 mM CN seawater a few minutes before onset of stimulation. This concentration of CN completely blocks respiration (13), and produces only about a 10% reduction in ATP during the first 20–30 min of exposure (8). This experiment complements the experiment in Fig. 14 in showing clearly that substrate, but not ATP, is necessary to energize calcium uptake by mitochondria.

Drs. Requena and DiPolo, in which ionized calcium was monitored by aequorin. The three axons with the highest loads clearly lie well above the other fibers. These three data are taken from the experiments illustrated in Figs. 11, 12, and 14 in which the fibers were stimulated at 100 impulses/s. This type of loading appears to affect buffering somewhat differently from soaking fibers in sodium-free solutions, because the rise during the first few minutes of stimulation is much more rapid (i.e. a rise of 3–10 μM) than it is during soaking. Actually, the rise during the linear portion of the loading curve is close to or somewhat less than the rise for injected or soaked fibers.

DISCUSSION

Calcium Contents of the FCCP or Cyanide-Sensitive and Insensitive Buffer Systems

The data presented in Results show that intact axons buffer an exogenous calcium load, however imposed, extraordinarily well. The mean rise in ionized calcium is about 0.6 nM/μM of load (i.e. better than 99.9% of a calcium load is buffered) for loads between 50 and 2,500 μM. The results have also shown that

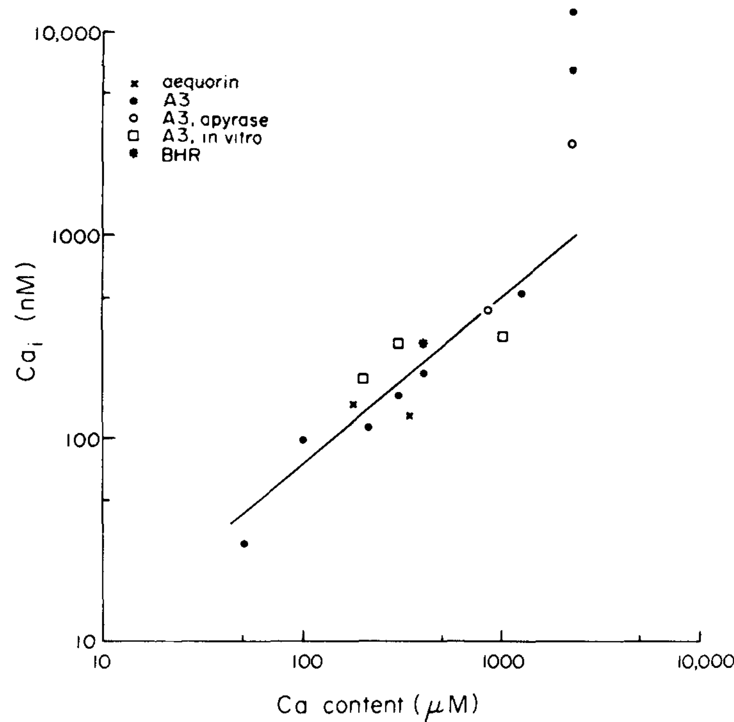

FIGURE 16. Collected data showing relation between ionized calcium and calcium content in intact squid axon. ×, Ionized calcium monitored by aequorin; ●, ionized calcium monitored by Arsenazo III; ○, Arsenazo III axon injected with apyrase; □, Arsenazo III in vitro experiment with axoplasm in quartz capillary; *, published datum point for stored axons of *L. forbesi* (2).

exogenous load is distributed between a system that is sensitive to either FCCP or cyanide, and one that is unaffected by these agents. In this section we present calculations that permit semiquantitative assignment of the calcium load to these systems. For convenience we designate the FCCP or cyanide-sensitive system the "M" buffer system implying, on the basis of the known sensitivity of mitochondrial calcium accumulation to these agents, that the "M" system probably is associated with mitochondria. The FCCP or cyanide-insensitive system we will call the "X" system, an identification which carries no implication as to the number or location of components of this buffer system.

In this Discussion it is assumed, simply for ease of calculation, that both the calcium content and ionized calcium are uniformly distributed throughout the

axoplasm. Although this probably occurs in the fresh unloaded axon, and may occur in isolated axoplasm after microinjection of a calcium load, uniform distribution of either form of calcium probably does not occur during calcium loading either by soaking in sodium-free solutions or by stimulation. Baker et al. (2) have calculated theoretical concentration profiles in squid axons and concluded that increases in ionized calcium due to increases in calcium influx produced changes in ionized calcium confined to the outer 50–100 μm of axoplasm. Applying these results to arsenazo-injected axons indicates that changes in ionized calcium measured by this technique can be regarded at best as mean steady-state values.

The increment in ionized calcium produced by FCCP in fresh axons was approximately 140 nM (Table I). Using the rounded figure, that any increment in ionized calcium seen under conditions of FCCP treatment is only about 5% of the calcium load, we can multiply this increment of 140 nM in ionized calcium by 20 to obtain the actual calcium released by the FCCP treatment. (The true multiplication factor is 16.7.) The 140-nM increment in ionized calcium therefore actually represents 2.8 μM calcium released when the M buffer system was inhibited by FCCP. We can use this result to fractionate the calcium content of fresh axons (50 μmol/kg axoplasm) into M and X buffer systems as follows:

$$\begin{aligned} \text{Calcium content} &= 50\ \mu\text{M} \\ \text{M buffer content} &= \underline{3\ \mu\text{M}} \\ \text{X buffer content} &= 47\ \mu\text{M}. \end{aligned}$$

Therefore we can conclude that in fresh intact axons containing only endogenous calcium, the M buffer system contains 3 μM or about 5% of the total calcium and the X buffer system(s) the other 95%.

As a second example of the calculation, we consider partitioning of the buffering capacity in a lightly loaded axon (Fig. 7). This axon, before FCCP treatment, was loaded with calcium by soaking the fiber for 12 min in a 1 mM Ca, 0 Na solution producing a calculated load of 48 μM. Addition of FCCP after this period of loading produced an increment in ionized calcium of about 850 nM. Using the datum on the buffering capacity of the X buffer system, we calculate that the total calcium sequestered in the M buffer system was 20 × 0.85 = 17 μM. The endogenous calcium, of course, contributed 3 μM to this total, leaving 14 μM taken up by the M buffer. Since the total load was 48 μM, the amount taken up by the X buffer system during the initial loading in 0 Na 1 mM Ca was 48 − 13 = 35 μM. For a slightly loaded axon, therefore, the M buffer system sequesters 13/48 or 27% of the exogenous load:

$$\begin{aligned} \text{Calculated calcium load} &= \phantom{17 - 4 = {}} 48\ \mu\text{M} \\ \text{Calcium taken up by M buffer} &= \underline{17 - 4 = 13\ \mu\text{M}} \\ \text{Calcium taken up by X buffer} &= \phantom{17 - 4 = {}} 35\ \mu\text{M}. \end{aligned}$$

Table III summarizes data on eight fibers in which an M- and X-buffered calcium could be determined, plus mean data from six fresh axons. The fraction of calcium load taken up by the M buffer system (column 8) ranged from a low of 5% (in fresh axons with no exogenous load) to a high of 50% in an axon with intermediate load. Table III also includes two axons injected with apyrase before

imposition of the exogenous load. Although apyrase per se does not affect mitochondrial buffering, it may affect distribution of calcium into the M and X buffer systems and explain why these two axons (each of which showed approximately 10% sequestration by the M buffer system) showed a lower M buffer content.

It is possible that the anomalously low amount of calcium apparently sequestered by the M buffer system in fresh axons (0 exogenous load, 50 μmol/kg axoplasm calcium content) may only reflect the fact that some of this 50 μM calcium is held in structural proteins and that the actual amount in the X system may be significantly lower than 47 μM.

TABLE III

DISTRIBUTION OF CALCIUM LOAD INTO X and M BUFFERS

1	2	3	4	5	6	7	8	9	10
Axon	Load ΔL	Content L	ΔX	X total	ΔM	M total	(col 7/ col 3) × 100	(col 6/ col 2) × 100	Ca_i
	μM	*μM*	*μM*	*μM*	*μM*	*μM*			*nM*
Fresh axon (n = 6)	0	50	0	47	0	3	5	–	30
5/19/76-1	20	70	10	57	10	13	19	50	50 (est.)
5/25/76-3	50	100	34	81	14	17	17	27	100
11/05/76-R	90	140	59	106	31	34	24	34	120
5/19/76-3	160	210	113	160	47	50	24	29	133
5/17/76-2	160	210	132	179	28	31	10	8	165
5/13/76-2*	318	368	298	345	20	23	6	6	–
5/18/76-3‡	800	850	775	822	75	79	9	9	430
AS-N-725	2,250	2,300	1,610	1,657	640	643	28	28	3,000

* In vitro axoplasm.
‡ Apyrase injection.

Intracellular Location of the "M" and "X" Buffer Systems

"M" BUFFER SYSTEM. The fact that a portion of the calcium-buffering capacity is FCCP sensitive has already been cited as evidence that mitochondria are involved in calcium buffering. Additional support for this hypothesis is derived from the magnitude of the buffering capacity. In heavily loaded fibers more than 2 mmol/kg axoplasm can be sequestered without exceeding the capacity of the axon. If the calcium were entering the mitochondria, the result would be understandable. Since mitochondria occupy about 1% of axon volume (12), a load of 2 mmol/kg axoplasm amounts to 200 mmol/kg mitochondria. Using a figure of 3.43 kg wet wt/kg protein,[1] a load of 200 mmol/kg mitochondria is equivalent to about 700 mmol/kg protein – a load which is well within the in vitro buffering capacity of mitochondria (20), even if all of it should go into these organelles.

[1] (*a*) Dry mass of mitochondria = 70% protein, 1 kg protein = $\frac{1}{0.7}$ = 1.43 kg dry wt. (*b*) Water content = 2 liter/kg protein = 1.40 liter/kg dry wt. (*c*) 1 kg dry wt mitochondria = 2.4 kg wet wt. (*d*) 1 kg protein = 1.43 × 2.4 = 3.43 kg wet wt.

"X" BUFFER SYSTEM(S). Since the arsenazo used as a calcium indicator in these experiments is itself a calcium buffer, the contribution of this dye to the nonmitochondrial buffering systems must be considered.

A significant contribution to the buffering capacity of intact or lightly loaded axons seems unlikely. FCCP treatment of fresh axons injected with arsenazo raised ionized calcium by 140 nM, equivalent to an approximately fivefold increase in free calcium. This is rather close to the results obtained in the preceding paper in which aequorin was used as a monitor of free calcium. With this technique metabolic inhibition by either cyanide or FCCP produced about a three-fold increase in light output.

If the limited increase in free calcium in arsenazo-injected axons resulted from binding to the dye, the increase in aequorin luminescence should have been very much larger.

In the case of heavily loaded axons, in which FCCP produces a large increase in ionized calcium, it seems likely that arsenazo is a significant part of the X buffering capacity. A more quantitative statement cannot be made until a way of measuring the spectrum and dissociation constant of the dye *in situ* is found.

Several possible locations for the components of the X buffer system may be excluded on quantitative grounds. It is unlikely that a significant amount of buffering occurs by soluble anions in the axoplasm. Table IV lists the major soluble calcium binding constituents in axoplasm together with estimates of their *in situ* binding constant in axoplasm. In aggregate these ions could complex only a few hundred nanomolar calcium, or less than 1% of that in the X buffer system of fresh axons, in the presence of a free ionized calcium of 30 nM.

Similarly, the calcium binding protein isolated by Alema et al. (1) from axoplasm of *L. vulgaris* can probably be excluded on quantitative grounds as a significant component of the X buffering system. The authors reported a mol wt

TABLE IV

ESTIMATES OF CALCIUM BINDING TO AXOPLASMIC CONSTITUENTS ($[Ca]_i$ = 30 nM, pH 7.3)

Ligand	Concentration in axoplasm	Effective dissociation constant*	[Ca-ligand]	Reference for dissociation constant
	mM	*mM*	*nM*	
Aspartate	80	~500	5	Tiffert and Brinley, unpublished observations (see reference 21)
Glutamate	20	~500	1	Assumed equal to aspartate (21)
ATP	4	0.7	171	Assume equal to K_{eff} for MgATP DeWeer, unpublished observations
ADP	~0.1	3.3	<1	Assume equal to K_{eff} for MgATP DeWeer, unpublished observations
AMP	~1	50	<1	Assume equal to K_{eff} for MgATP DeWeer, unpublished observations
Phosphate	~3	35	3	(24)
Ca binding protein	0.18	0.025	216	(1)
Ca binding protein	0.03	0.0005	1,800	(3)

* Minimum estimate, would be increased in presence of Mg_i.
Ionic strength ~0.3.

of 13,500 for this protein, which is present in the axoplasm of *L. vulgaris* at a concentration of 2.40 g/kg wet wt (i.e. 180 μM). This protein has two binding sites under physiological conditions and a dissociation constant of 25 μM. It could therefore hold only ~70 nM calcium at physiological free calcium concentrations, and not more than 360 μM at saturation. Since the present experiments show that the axon can buffer at least 2,250 μM calcium without any signs of saturation, the capacity of this protein would seem inadequate for buffering large loads, and its dissociation constant too large to hold significant calcium in fresh axons.

Baker and Schlaepfer (3) have reported a calcium binding entity insensitive to 2 mM cyanide and oligomycin, with a dissociation constant of about 0.5 μM and a capacity of 35–50 μM. This substance probably could bind no more than a few micromolar of calcium in fresh axoplasm, therefore it is unlikely to be the major part of the X buffer system in fresh axons, although it could be a significant calcium binding entity for lightly loaded axons which have loads of less than 50 μM.

It is possible that components of the X buffer system are located in organelles, located immediately subjacent to the axolemma. Oschman et al. (22) have demonstrated the existence of deposits of calcium phosphate immediately underneath the axolemma which form when squid axons are fixed in glutaraldehyde solution containing 5 mM calcium. Similar deposits were seen in squid axons by Hillman and Llinás (15). Henkart (14) has described reticulum-like structures subjacent to the axolemma which swell under conditions in which calcium content of the axoplasm increases, suggesting that they may be taking up calcium with a resultant osmotic swelling.

One of the components of the X buffer system may be structurally associated with the M buffer system. This possibility arises from a consideration of the behavior of the buffer systems during heavy loading of the fiber by stimulation. After an initial rapid rise of several micromolar of ionized calcium immediately after stimulation is begun, the subsequent rise is very slow, at the rate of 0.2 or 0.3 nM/μM load. Since the evidence indicates clearly that the X buffer system cannot buffer so much ionized calcium in the presence of such a calcium load (approximately 2.5 mM total calcium load), the assumption is that this extreme degree of buffering involves the M buffer system. This circumstance would seem to require that nearly the entire load (i.e. about 2.5 mM) of calcium passes into the M buffer system during the loading process. However, calculation of the load in the M buffer system, in Fig. 12, at the end of the loading amounted to only 600 μM, although the calculated load that passes into the axon after the plateau of ionized calcium is reached (5 min after stimulation) is almost the total load.

The simplest ad hoc hypothesis to reconcile these apparently contradictory experimental findings is to suppose that the M buffer system can take up more calcium than it can ultimately hold in a labile form. Some of the calcium passes into a more stable form which would not be FCCP sensitive and which in the operational definition of this paper would be classified as part of the X buffer system. There are two pieces of in vitro evidence which support this speculation: (*a*) maximally effective concentrations of FCCP do not release all of the mito-

chondrial calcium (25); (*b*) there is evidence (20) that in the presence of extramitochondrial phosphate in concentrations which occur in squid axoplasm (ca. 1 mM) some of the calcium accumulated in mitochondria during heavy loading in vitro is held in the form of hydroxyapatite which is poorly soluble and would release calcium very slowly over the time scale of our experiments.

Ratio of Intra- and Extramitochondrial Calcium Concentration

Rottenberg and Scarpa (25) have calculated the nominal intramitochondrial calcium concentration using the FCCP releasable calcium as a measure of the metabolically labile calcium held within the mitochondria. It is interesting to repeat this calculation for squid axons, assuming that the M buffer system represents calcium held in free form within mitochondria. The amount of endogenous FCCP-releasable calcium has already been calculated to be 2.8 μmol/kg axoplasm, if one takes into account the effect of buffering by the X system. Since the mitochondria occupy 1% of axon volume, the calcium releasable from the M system, if confined to the mitochondria, would have an apparent concentration of 280 μmol/kg mitochondria, or roughly 280 μM. If the ambient free Ca in the axoplasm is taken as 30 nM, the ratio of intra- to extramitochondrial calcium concentration is 9,000 for fresh axons.

Data for all experiments with varying total loads are presented in Table V. The table shows concentration ratios of 9,000 for fresh axons, 20,000 for lightly loaded axons, and 6,400 in a single, heavily loaded axon. This latter figure, for an axon loaded with calcium until the intramitochondrial calcium is in a near steady state with an extramitochondrial calcium of 10 μM, is reasonably close to the value reported by Scarpa and Rottenberg (4,000–5,000) for ratios in rat liver mitochondria which were in equilibrium with somewhat higher extramitochondrial calcium concentrations (~50 μM), suggesting that similar processes for sequestering calcium occur in vitro and *in situ* for the FCCP-sensitive buffer system.

Mitochondrial Calcium Content and Concentrations

In fresh squid axons the FCCP-releasable calcium amounts to 2.8 μmol/kg axoplasm or 0.28 mmol/kg mitochondria. Using commonly accepted data on

TABLE V

CALCIUM CONCENTRATION RATIO IN SQUID MITOCHONDRIA

1 Axon	2 Ca_i	3 Ca in M buffer	Ratio Col. 3 / Col. 2
	nM	*µM*	
Fresh axons (n = 6)	30	280	9,000
11/05/76-B	120	3,400	28,330
5/25/76-3	100	1,700	17,000
5/19/76-3	133	4,700	35,000
5/18/76-2	430	7,500	17,440
5/13/76-2	368	2,000	5,430
5/17/76-2	165	2,800	17,000
5/19/76-1	70	1,400	20,000
AS-N-725	10,000	64,000	6,400

mitochondrial water and protein content, one can calculate that the labile calcium in the mitochondria is equivalent to $0.28 \times 3.43 = 1$ mmol calcium/kg mitochondrial protein. This value is much lower than the usual published values for calcium content in mitochondria freshly isolated from brain, liver, and kidney which range from 10 to 15 mmol/kg protein (mitochondria isolated from smooth muscle can have a much higher calcium content).

An upper limit to the total calcium content obtained by assuming that all of the calcium in fresh axoplasm (50 μmol/kg axoplasm) is in the mitochondria gives a Ca content of about 17 mmol/kg protein. Although this latter figure is closer to that of isolated mitochondria, the agreement may be fortuitous. In vitro preparation of mitochondria necessarily involves exposure to extracellular calcium no matter how carefully the preparation is washed, and this circumstance could permit substantial uptake of exogenous calcium. On the other hand, some intramitochondrial calcium could be lost during the EDTA washes that customarily accompany the final stages of mitochondrial isolation.

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REFERENCES

1. ALEMA, S., P. CALISSANO, G. RUSCA, and A. GIUDITTA. 1973. Identification of a calcium-binding, brain specific protein in the axoplasm of squid giant axons. *J. Neurochem.* **20**:681–689.
2. BAKER, P. F., A. L. HODGKIN, and E. B. RIDGWAY. 1971. Depolarization and calcium entry in squid axons. *J. Physiol. (Lond.).* **218**:709–755.
3. BAKER, P. F., and W. SCHLAEPFER. 1975. Calcium uptake by axoplasm extruded from giant axons of *Loligo. J. Physiol. (Lond.).* **239**:37P–39P.
4. 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.
5. BRINLEY, F. J., JR., and L. J. MULLINS. 1965. Ion fluxes and transference numbers in squid axons. *J. Neurophysiol.* **28**:526–544.
6. BRINLEY, F. J., JR., and A. SCARPA. 1975. Ionized magnesium concentration in axoplasm of dialyzed squid axons. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **50**:82–85.
7. BRINLEY, F. J., JR., A. SCARPA, and T. TIFFERT. 1977. The concentration of ionized magnesium in barnacle muscle fibers. *J. Physiol. (Lond.).* **266**:545–565.
8. CALDWELL, P. C. 1960. The phosphorous metabolism of squid axons and its relationship to the active transport of sodium. *J. Physiol. (Lond.).* **152**:545–560.

8*a*. CARAFOLI, E., and M. CROMPTON. 1976. Calcium ions and mitochondria. *In* Calcium in Biological Systems. C. J. Duncan, editor. Cambridge University Press.

9. CONNELLY, C. M., and P. F. CRANEFIELD. 1953. The oxygen consumption of the stellar nerve of the squid (*Loligo pealei*). XIX International Physiological Congress, Montreal.
10. DEFFNER, G. G. J. 1961. The dialyzable free organic constituents of squid blood; a comparison with nerve axoplasm. *Biochim. Biophys. Acta.* **47**:378–388.
11. DE WEER, P. 1970. Effects of intracellular adenosine-5'-diphosphate and orthophos-

phate on the sensitivity of sodium efflux from squid axon to external sodium and potassium. *J. Gen. Physiol.* **56:**583–620.

12. 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.
13. Doane, M. G. 1967. Fluorometric measurement of pyridine nucleotide reduction in the giant axon of the squid. *J. Gen. Physiol.* **50:**2603–2632.
14. Henkart, M. 1975. The endoplasmic reticulum of neurons as a calcium sequestering and releasing system: morphological evidence. *Biophys. Soc. Abstr.* 267*a*.
15. Hillman, D. E., and R. Llinás. 1974. Calcium-containing electrondense structures in the axons of the squid giant synapse. *J. Cell. Biol.* **61:**146–155.
16. Hodgkin, A. L., and R. D. Keynes. 1956. Experiments on the injection of substances into squid giant axons by means of a microsyringe. *J. Physiol.* (*Lond.*). **131:**592–616.
17. Hodgkin, A. L., and R. D. Keynes. 1957. Movements of labelled calcium in squid giant axons. *J. Physiol.* (*Lond.*). **138:**253–281.
18. Keynes, R. D., and P. R. Lewis. 1956. The intracellular calcium contents of some invertebrate nerves. *J. Physiol.* (*Lond.*). **134:**399–407.
19. LaNoue, K. F., J. Bryla, and D. J. P. Bassett. 1974. Energy driven aspartate efflux from heart and liver mitochondria. *J. Biol. Chem.* **249:**7514–7521.
20. Lehninger, A. L. 1970. Mitochondria and calcium ion transport. *Biochem. J.* **119:**129–138.
21. Lumb, R. F., and A. E. Martell. 1953. Metal chelating tendencies of glutamic and aspartic acids. *J. Phys. Chem.* **57:**690–693.
22. Oschman, J. L., T. A. Hall, P. D. Peters, and B. J. Wall. 1974. Association of calcium with membranes of squid giant axons. *J. Cell. Biol.* **61:**156–165.
23. Requena, J., R. DiPolo, L. J. Mullins, and F. J. Brinley, Jr. 1977. The control of intracellular calcium concentration in squid axons. *J. Gen. Physiol.* **70:**329–353.
24. Ringbom, A. 1963. Complexation in Analytical Chemistry. Interscience Publishers, Div. of J. Wiley & Sons, New York, London.
25. Rottenberg, H., and A. Scarpa. 1974. Calcium uptake and membrane potential in mitochondria. *Biochemistry.* **13:**4811–4817.
26. Scarpa, A. 1972. Spectrophotometric measurement of calcium by murexide. *Methods Enzymol.* **24:**343–351.