Calcium Efflux from Internally Dialyzed Squid Giant Axons

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ABSTRACT Calcium efflux has been studied in squid giant axons under conditions in which the internal composition was controlled by means of a dialysis perfusion technique. The mean calcium efflux from axons dialyzed with 0.3 μM calcium and 5 mM ATP was 0.26 pmol/cm^2·s at 22°C. The curve relating the Ca efflux with the internal Ca concentration had a slope of about one for $[Ca]_i$ lower than 0.3 μM and a slope smaller than one for higher concentrations. Under the above conditions replacement of $[Na]_o$ and $[Ca]_o$ by Tris and Mg causes an 80% fall in the calcium efflux. When the axons were dialyzed with a medium free of ATP and containing 2 mM cyanide plus 5 μg/ml oligomycin, analysis of the perfusion effluent gave values of 1–4 μM ATP. Under this low ATP condition, replacement of external sodium and calcium causes the same drop in the calcium efflux. The same effect was observed at higher $[Ca]_i$, (80 μM). These results suggest that the Na-Ca exchange component of the calcium efflux is apparently not dependent on the amounts of ATP in the axoplasm. Axons previously depleted of ATP show a significant transient drop in the calcium efflux when ATP is added to the dialysis medium. This effect probably represents the sequestering of calcium by the mitochondrial system. The consumption of calcium by the mitochondria of the axoplasm in dialyzed axons was determined to be of the order of 6.0×10^{-7} mol Ca^{++}/mg of protein with an initial rate of 2.6×10^{-8} mol Ca^{++}/min·mg of protein. Axons dialyzed with 2 mM cyanide after 8–10-min delays show a rise in the calcium efflux in the presence of "normal" amounts of exogenous ATP. This effect seems to indicate that cyanide, per se, can release calcium ions from internal sources.

INTRODUCTION

Under normal circumstances, living cells contain extremely low concentrations of ionized calcium. More than one mechanism seems to be involved in maintaining this low calcium cytoplasmic concentration. For instance, red cells which lack intracellular organelles can keep a low internal calcium concentration by a mechanism in which the hydrolysis of ATP provides the energy for extrusion of calcium across the plasma membrane (Schatzmann and Vincenzi, 1969). In other types of cells, the presence of intracellular organelles

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(mitochondria, endoplasmic reticulum, etc.) can provide a means to control the internal calcium concentration in view of the ability of these organelles to accumulate calcium ions by processes depending on electron transport and/or ATP hydrolysis.

In all cases, however, the balance of calcium fluxes across the cellular membrane should be the determining factor in maintaining the low intracellular calcium concentration. In erythrocytes and cultured cells (Schatzmann and Vincenzi, 1969; Borle, 1969; and Lamb and Lindsay, 1971) this flux balance seems to depend in a direct manner on ATP hydrolysis. Recently a new mechanism for extruding calcium has been described by Reuter and Seitz (1968) in cardiac muscle, by Baker et al. (1969), and Blaustein and Hodgkin (1969) in squid axons, suggesting that calcium extrusion is coupled with an inward downhill movement of sodium ions. This sodium-calcium exchange mechanism has also been confirmed in a variety of other tissues, most of them excitables (Baker and Blaustein, 1968; Kalix, 1971; DiPolo, 1973).

In view of the experimental evidences for sodium-calcium counter transport, the question arises as to the source of energy for this mechanism. Blaustein and Hodgkin (1969) have suggested exchange schemes to explain the experimental equilibrium ratio $[Ca_o]/[Ca]_i$, based on a simple sodium-calcium exchange. However, one piece of evidence is needed in order to consider the validity of such models. That is, whether a specific energy source, such as ATP hydrolysis, can drive this exchange of calcium for sodium. The metabolic inhibitors, cyanide and dinitrophenol, act by preventing the generation of high energy phosphate bonds by oxidative metabolism. The application of such substances to squid axons has been shown by Caldwell et al. (1960) to reduce the ATP concentration in the axoplasm to about 10% normal. However, under this low ATP condition, the sodium pump is not fully inhibited since the experimental sodium efflux is seven times larger than that predicted for a purely passive outward lead of sodium (Mullins and Brinley, 1967). Using a dialysis technique, Mullins and Brinley (1967) have been able to reduce the ATP concentration of the axoplasm to about 1–10 μM. At this ATP concentration the remaining sodium efflux could be safely calculated from the measured sodium influx and the resting membrane potential.

The experiments described here were undertaken in order to determine whether a sodium-calcium counter transport could be maintained in the absence of exogenous ATP achieved experimentally by the dialysis technique. A second phase of the work was concerned with measurements of the calcium consumption in vivo by the mitochondrial system of the axon, as well as with the effect of cyanide on the calcium released from intracellular stores in the presence of exogenous ATP.

The general conclusion from this work is that dialyzed axons show a significant sodium-dependent calcium efflux in the apparent absence of ATP.

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METHODS

The experiments reported here were performed using freshly caught live specimens of *Dorytheutis plei*. After decapitation the hindmost giant axon from the stellate ganglion was dissected from the mantle in flowing sea water and cleaned of connective tissue under a dissecting microscope. The mean axon diameter was 440 ± 50 μm ($n = 20$).

Solutions

The solutions used in this study are given in Table I. Radioactive dialysis solutions were made by adding solid $Ca^{45}Cl_2$ (International Chemical and Nuclear Corporation, Irvine, Calif. 20 mCi/mg) directly to the perfusion solution. The Na, K, and

TABLE I

SOLUTIONS

Substance	ASW	Ca-free LiASW	Internal dialysis*
	mM	*mM*	*mM*
Na^+	442	—	80
Li^+	—	442	—
K^+	10	10	335
Mg^{++}	53	64	4
Ca^{++}	11	—	—
Cl^-	590	590	90
Isethionate$^-$	—	—	335
$Tris^+$	10	10	10
Sucrose	—	—	200

* Described in text as "fuel-free"; additions made were ATP 5 mM. Internal Mg^{++} was set at 4 mM in excess of [ATP]. NaCN and oligomycin when added were used at 2 mM and 5 μg/ml, respectively. EGTA when used was set at (0.47 mM), pH 7.1.

Tris salts of ATP were purchased from Sigma Chemical Co., St. Louis, Mo. They were dissolved in appropriate amounts of KOH so that the resulting solution was 0.5 M. The pH was adjusted to 7.0. Stock solutions of ATP were stored at −25°C. NaCN, oligomycin, and EGTA were obtained from Sigma Chemical Co.

Biochemical Procedures

ATP ANALYSIS ATP analyses were done by the firefly flash method previously modified by Mullins and Brinley (1967). Amounts as low as 10^{-12} mol of ATP could be measured with enough accuracy. Measurements of ATP concentrations in the dialysis effluent were made by a calibrated glass capillary tube that was positioned over the end of the dialysis capillary. Fluid samples had volumes from 2 to 7 μl. Upon collection, the samples were stored at −25°C and measured for ATP content at the end of the experiment.

MITOCHONDRIAL ANALYSIS To determine the amount of mitochondrial protein in the axoplasm, samples of axoplasm were extruded into capillary tubes of known

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volumes and the axoplasm was subjected to three successive centrifugations at 25,000 *g* for 15 min at 4°C. Electron microscopy studies using a negative-stain technique confirmed that the experimental procedure was adequate to remove the mitochondria from the axoplasm. The resulting mitochondrial pellet was suspended in 0.25 M sucrose. This suspension contained approximately 0.7 mg protein/ml axoplasm measured by the method of Lowry et al. (1951).

In order to study the distribution and geometry of mitochondria in the axoplasm, nerve fibers were analyzed by electron microscopy using transversal and longitudinal sections. It was observed that in this squid species the mitochondria were uniformly distributed in the axoplasm. This homogeneous distribution of mitochondria indicates that the axoplasmic samples used to determine the mitochondria protein content of the axoplasm were indeed representative of the concentration of mitochondria in the axoplasm. The cross-sectional area occupied by the mitochondrial compartment was estimated in a series of transverse sections and expressed as a fraction of the total transverse area. From the longitudinal sections it was possible to get the volume of the mitochondrial compartment which amounts to an average of about 1% of the axoplasmic volume.

Internal Dialysis

The apparatus and procedure to dialyze isolated squid axons have been previously described (Brinley and Mullins, 1967). The porous glass capillary used in the present study had an outside diameter of about 125 μm. The porosity of these capillaries as supplied by the manufacturer (Corning Glass Works, Corning, N.Y.) could be easily increased by simply soaking them in 60 mM Na-EDTA (pH 6.5) for 6–12 h at room temperature. To free axoplasm from a desired solute the axoplasm was routinely dialyzed at both left- and right-hand junctions of the porous-nonporous glass (see Mullins and Brinley, 1967).

The calcium contamination (mainly due to the K isethionate) in the dialysis solution was measured by atomic absorption spectrophotometry. It ranged between 50 and 80 μM. Therefore, a calcium buffering system (EGTA) had to be used for the low internal calcium solutions. The ionized calcium present in the calcium buffering solutions was calculated according to Portzehl et al. (1964).

RESULTS

Resting Calcium Efflux

The mean efflux of calcium from four axons bathed in normal artificial seawater (ASW) at 22°C and dialyzed with a fluid containing 5 mM ATP and 0.3 μM of calcium was 0.26 pmol/cm^2·s. The time necessary to reach a steady ^{45}Ca efflux from the onset of the dialysis ranged from 30 to 50 min as compared with a time of 8–9 min observed for reaching a steady efflux with ^{22}Na (DiPolo, unpublished observation).

Dependence of Calcium Efflux on Internal Calcium Concentration

Fig. 1 shows the results of three experiments carried out to obtain information on the effect of internal free calcium on the calcium efflux in the range be-

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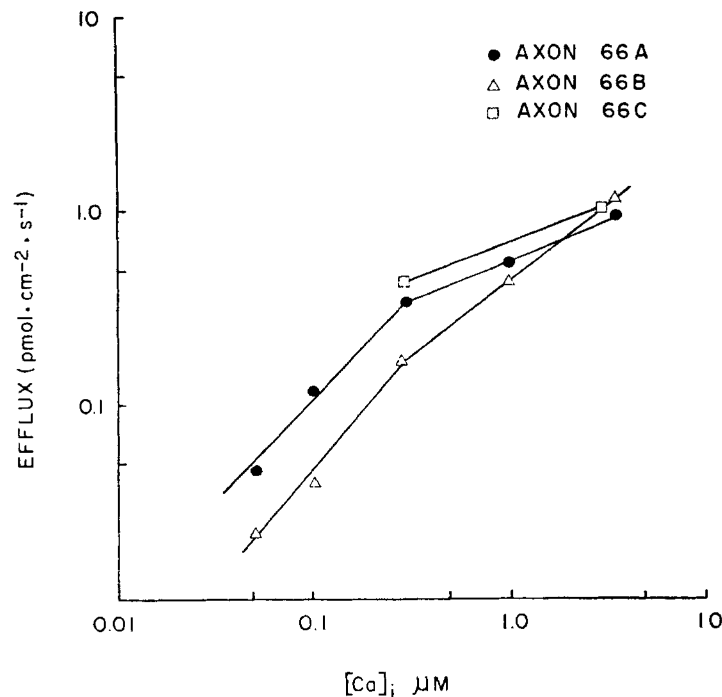

FIGURE 1. Calcium efflux as a function of the internal ionized calcium concentration on double logarithmic scale. Abscissa: time in min. Ordinate: calcium efflux in pmol/ $cm^2 \cdot s$. The dialysis solution contains 5 mM ATP; the external medium was ASW. Buffer Ca-EGTA was used to adjust the internal ionized calcium.

tween 0.05 and 3 μM. For these experiments the free calcium was varied by changing the amount of calcium added to the perfusion mediums at constant EGTA concentration. The dialysis fluid contained ATP and no poisons were added.

For $[Ca]_i$ lower than 0.3 μM the slope of the curve relating Ca efflux to internal free calcium is about one on a double logarithmic scale. For concentrations higher than 0.3 μM the slope seems to be smaller than one, which might indicate the initial portion of a saturation curve. Higher calcium concentrations were used in other experiments as will be reported later. The efflux values obtained with these higher $[Ca]_i$ have not been included in Fig. 1, since they were obtained under different experimental conditions.

Calcium Sodium Exchange in the Absence of ATP

In order to measure the effect of external sodium and calcium on the calcium efflux in the absence of high energy phosphate compounds, a number of axons were pretreated with 2 mM cyanide for 1 h before beginning the experiment. As shown by Caldwell et al. (1960), this procedure reduces the ATP of axoplasm to about 120 μM. The subsequent dialysis of the axoplasm with fuel-

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free fluid, yielded a concentration of ATP in the dialysis effluent of about 1–4 μM. Oligomycin, a specific inhibitor of phosphorylating respiration, was added to the dialysis fluid in a concentration of 5 μg/ml. Fig. 2 shows a typical experiment in which the calcium efflux was measured in normal ASW containing $[Na]_o = 442$ and $[Ca]_o = 11$ mM. The internal dialysis fluid was fuel-free and contained cyanide. The $[Ca]_i$ was buffered at 0.27 μM with EGTA. Under these conditions, and after a base line for calcium efflux has been established, the external ASW was changed for one containing 0 Na and 0 Ca. This procedure promptly reduced the calcium efflux to about 20% of its

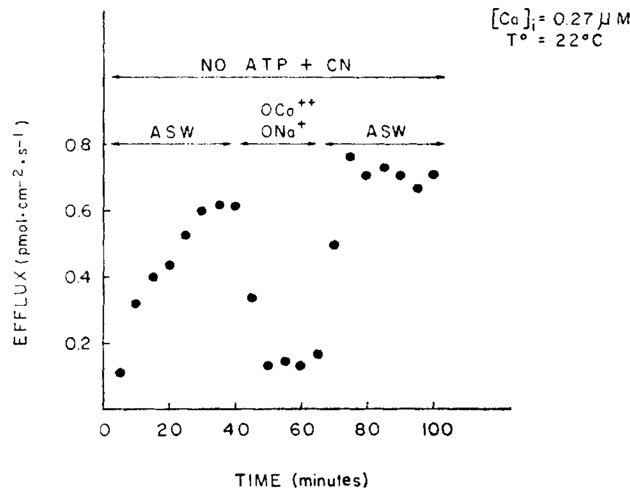

FIGURE 2. The effect of external sodium and calcium on the calcium efflux in an axon dialyzed with fuel-free, 2 mM CN^-. The external medium was ASW containing 2 mM CN^-. Abscissa: time in min. Ordinate: calcium efflux in pmol/cm^2·s. In this experiment the axon was predialyzed for 45 min with fuel-free, 2 mM CN^- medium before adding the radioactive ^{45}Ca. The [ATP] in the axoplasm during the steady state flux was 3 μM. Axon diameter 495 μm.

initial value; it is apparent then, that under these circumstances, most of the calcium efflux can be accounted for by an exchange with external sodium and calcium. Fig. 3 shows that the same effect was observed in the absence of Ca chelating agents, a condition which implies a much higher $[Ca]_i$. One useful piece of information that was easily obtained by the internal dialysis technique was the effect of ATP on the low level calcium efflux, in the absence of external sodium and calcium. Fig. 3 shows that a subsequent change in the dialysis fluid to one containing ATP at 5 mM had a small but significant effect on the level of the calcium efflux. Restoration of normal sodium and calcium in the external medium caused an increase in the calcium efflux, showing that the effect described is reversible. Similar results were obtained with four different axons. It is interesting to point out that in poisoned axons, independently of

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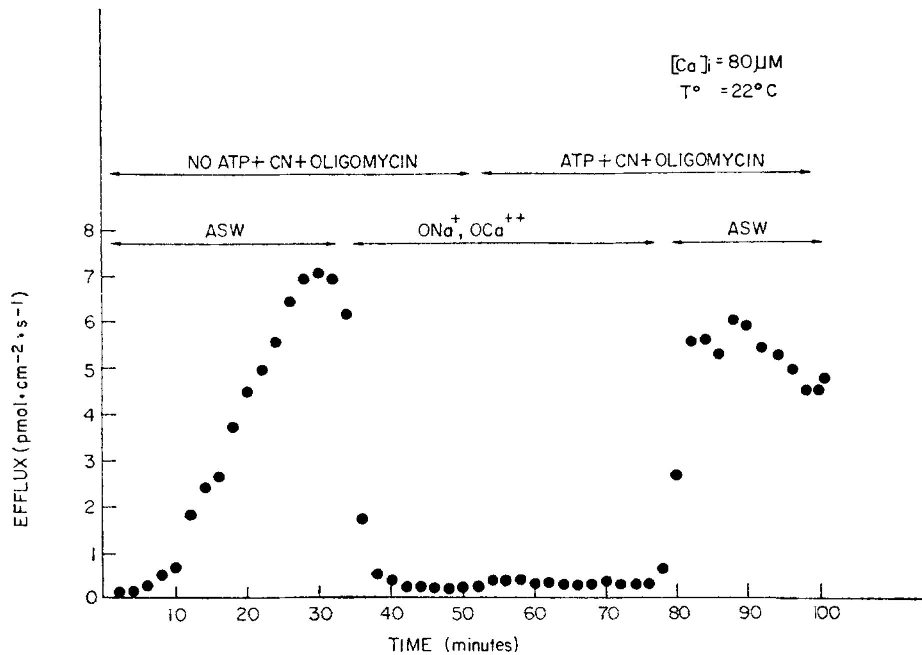

FIGURE. 3. The effect of external sodium and calcium on the calcium efflux in an axon dialyzed with fuel-free 2 mM CN^- and 5 μg/ml of oligomycin medium in the absence of EGTA buffer. Abscissa: time in min. Ordinate: calcium efflux in pmol/$cm^2 \cdot s$. The [ATP] in the axoplasm during fuel-free dialysis was 2 μM. Axon diameter 420 μm.

the internal calcium concentration, the calcium efflux values were higher than in nonpoisoned axons.

Calcium Efflux in the Absence of Cyanide and Oligomycin

In the experiment described above, cyanide and oligomycin were included in the dialysis fluid. These drugs are known to block the active uptake of calcium by the mitochondrial system. It was thought of interest to test to what extent the mitochondrial compartment serves as a regulating system for the intracellular calcium. In order to do so, EGTA buffer had to be excluded from the perfusion medium so that changes in the internal free calcium concentration could take place and their effect on the calcium efflux could be observed. Fig. 4 shows the effect of ATP on the calcium efflux in a nonpoisoned axon. In this and similar experiments axons were predialyzed without ATP for about 20 min before supplying radioactive calcium to the porous capillary. After the calcium efflux had reached a steady level in the absence of ATP, changing the internal perfusion medium to one containing 5 mM ATP caused a marked initial drop in the calcium efflux, followed by a slow increase in the efflux. When the ATP was removed from the dialysis fluid a sudden rise in the Ca efflux occurred reaching a higher value than that of the original resting flux.

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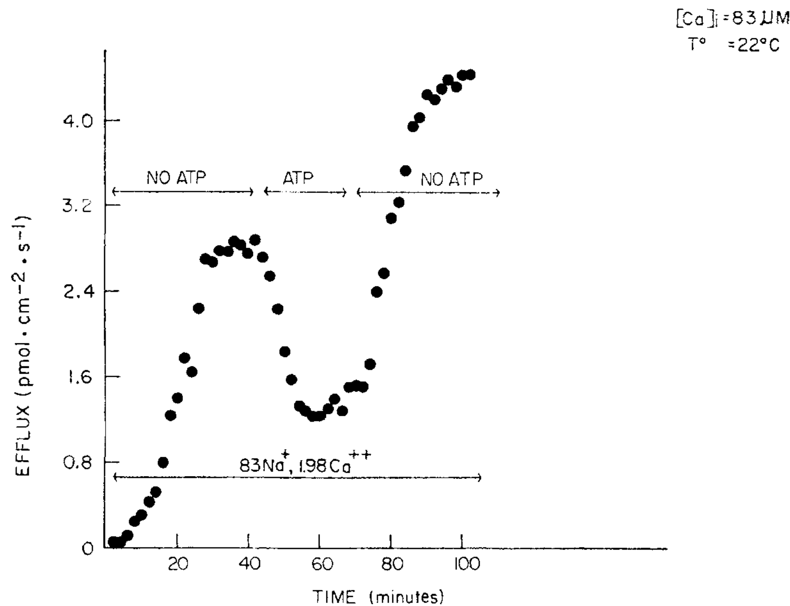

FIGURE 4. The effect of ATP at 5 mM on the calcium efflux in unpoisoned axon. Abscissa: time in min. Ordinate: calcium efflux in pmol/cm²·s. The [ATP] in the axoplasm during fuel-free dialysis was 12 μM. Axon diameter 400 μm.

Similar results have been obtained by Baker et al. (1971) by injecting ATP into cyanide-poisoned axons. Theoretical calculations using the association constants for Ca-ATP and Mg-ATP complexes, indicate that in the presence of a ratio $[Mg^{++}]/[Ca^{++}] = 3{,}000$, only a very small fraction of the total ionized calcium is in the form of a Ca-ATP complex. This point was checked experimentally by measuring the changes in light absorption which occur when calcium complexes with murexide. The medium contained the above-mentioned $[Mg^{++}]/[Ca^{++}]$ ratio to which ATP was added or omitted. No significant differences were observed in the presence or absence of ATP. This suggests that the observed decrease in the calcium efflux in the presence of ATP is not due to a decrease in the ionized Ca^{++} in the form of a Ca-ATP complex. The most likely explanation for this phenomenon is that in the absence of exogenous ATP the mitochondrial system partially loses its ability to accumulate calcium. Therefore, when ATP is added to the perfusion medium there is a significant drop in calcium concentration in the axoplasm as a result of its active accumulation by the mitochondria. The active transport of calcium by the mitochondrial system could also explain the marked increase in calcium efflux observed when ATP is removed at the end of the experiment, since in the absence of ATP the mitochondria will liberate a sizable fraction of endogenous calcium.

Although the mitochondrial system could account for the effect of ATP on

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the calcium efflux, one cannot rule out, a priori, a direct specific effect of ATP on the calcium permeability at the axon membrane level. The dialysis technique offers a convenient way to clarify this point. In fact, it is possible to determine the amount of calcium coming out in the dialysis effluent via the porous capillary, as well as that leaving the axon via the membrane. Therefore, if the drop in the calcium efflux observed when ATP is added to the dialysis medium is due to the buffer effect of the mitochondrial system, one should expect a decrease in the calcium concentration in the dialysis effluent. On the other hand, if ATP is acting by decreasing the calcium permeability of the axon membrane, there should be either no change or a small increase in the calcium concentration in the dialysis effluent. In Fig. 5 both the calcium efflux and the calcium concentration in the dialysis are shown. It is clear that in the presence of ATP at 5 mM, a drop in both calcium efflux and calcium concentration in the dialysis effluent are observed. It is interesting to note that in both curves the effect of ATP appears to be of a transient nature, both curves increasing slowly with time at about the same rate.

Further evidences of the buffer capacity of the mitochondrial system were obtained by extruding the axoplasm of intact axons into small (900-μm diameter and 3–4-cm long) glass capillaries. The glass capillaries containing the axoplasm were dialyzed with the standard dialysis technique using a perfusion solution with or without ATP. Fig. 6 shows the effect of adding ATP

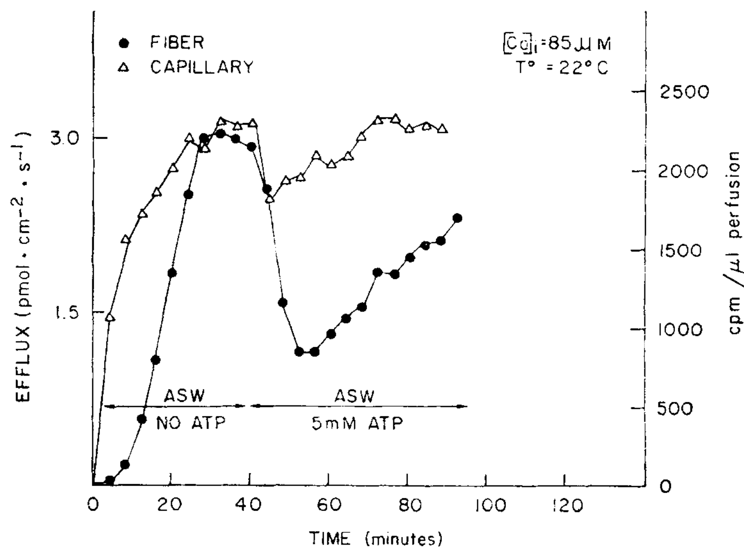

FIGURE 5. The effect of ATP at 5 mM on both the calcium efflux from the axon and the calcium concentration from the dialysis effluent in the unpoisoned axon. Abscissa: time in min. Ordinate: ● is the calcium efflux in pmol/cm²·s; △ concentration of calcium in the dialysis effluent in cpm/μl perfusion. The [ATP] in the axoplasm during fuel-free dialysis was 15 μM. Axon diameter 475 μm.

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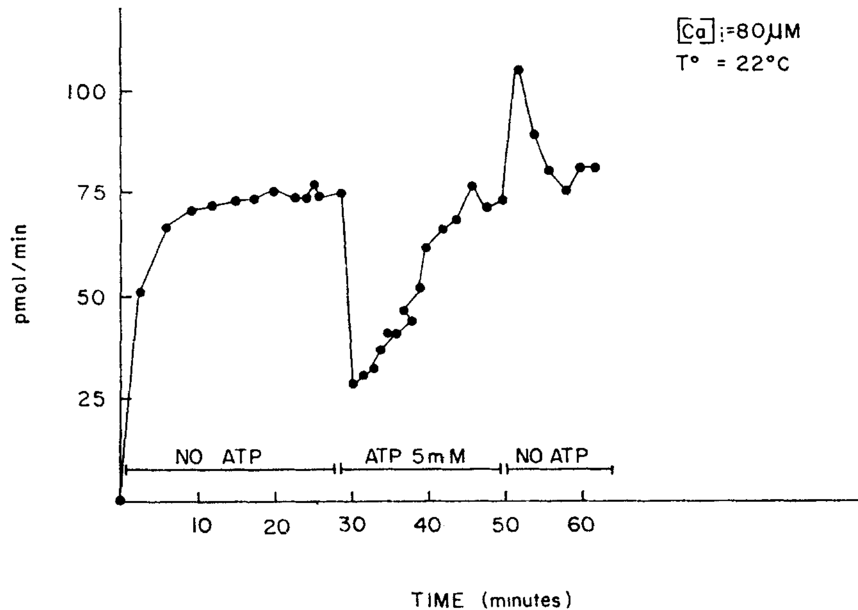

FIGURE 6. The effect of ATP on the calcium concentration in the dialysis effluent of a dialyzed sample of axoplasm. Abscissa: time in min. Ordinate: calcium flux in the dialysis effluent in pmol/min. Volume of extruded axoplasm = 5 μl.

at 5 mM to the calcium concentration of the dialysis effluent of the dialyzed axoplasm which previously had been perfused without ATP for almost 30 min. A rapid transient fall is observed in the calcium effluent. The $t_{\frac{1}{2}}$ of saturation for this particular experiment was of the order of 10 min. A subsequent change in the dialysis solution to one containing no ATP led to a transient increase in the calcium concentration in the porous capillary. The two transient changes in the calcium concentration in the porous capillary most probably represent the uptake and release of calcium from the mitochondria in the presence and in the absence of ATP. It should be pointed out that a great experimental variability was observed in these dialyzed axoplasm experiments. Blaustein and Hodgkin (1969) have reported that the calcium efflux from extruded axoplasm slowly drifted up to a high value. Presumably the lability of the mitochondrial system, as well as the loss of a substrate from the dialyzed axoplasm could explain in part the experimental variations.

Effect of Cyanide on the Calcium Efflux

The respiratory inhibitor, cyanide, has been shown by Rojas and Hidalgo (1968) and Blaustein and Hodgkin (1969) to increase markedly the calcium efflux from squid axons. This substance, which reduces the levels of ATP to about 100 μM, probably causes a release of bound calcium from the mitochondria with the concomitant rise in the calcium efflux. Cyanide has been

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used to test whether the increase in the calcium efflux can be accounted for solely by the low ATP levels present in these axons or whether cyanide, per se, can liberate part of the bound calcium in the presence of normal amounts of ATP. An experiment of this sort is shown in Fig. 7. In the first part of the experiment it is shown that partially lowering the external sodium and calcium concentrations in an axon dialyzed with 5 mM ATP caused a marked reduction in the calcium efflux which could be reversed by restoring the initial external conditions. Since the primary purpose of this experiment was to determine the effect of cyanide in the presence of ATP, the internal dialysis medium was changed to one containing ATP at 5 mM and cyanide at 2 mM. As can be seen in Fig. 7, a prompt (8–10-min delay) rise in the calcium efflux to a level about 4–5 times the resting level was obtained. This increase in calcium efflux was unlikely to be a simple leak, since partially removing the external sodium and calcium caused the same percent inhibition on the calcium efflux as that observed in the absence of cyanide. The effect of external sodium and calcium in this cyanide axon, suggests that the increase in Ca efflux is mostly an exchange for external sodium and calcium. Similar results were obtained with three different axons, although the increase in Ca efflux varied from three- to sevenfold. Another experiment of the effect of cyanide is shown in

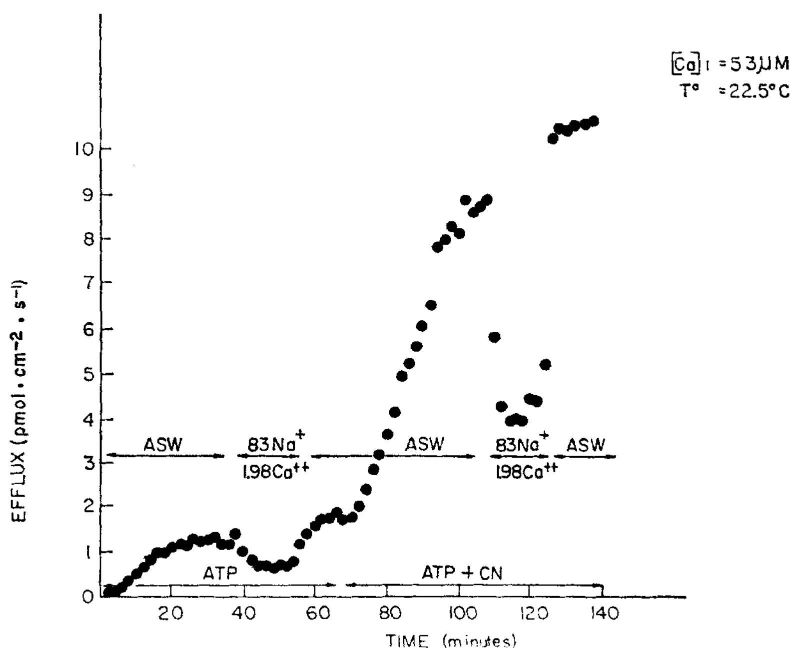

FIGURE 7. The effect of internal cyanide at 2 mM on the calcium efflux in the presence of 5 mM ATP throughout the experiment. On two occasions during the experiment the external sodium and calcium were reduced to 83 and 1.98 mM, respectively. Abscissa: time in min. Ordinate: calcium efflux in pmol/cm²·s. Axon diameter 450 μm.

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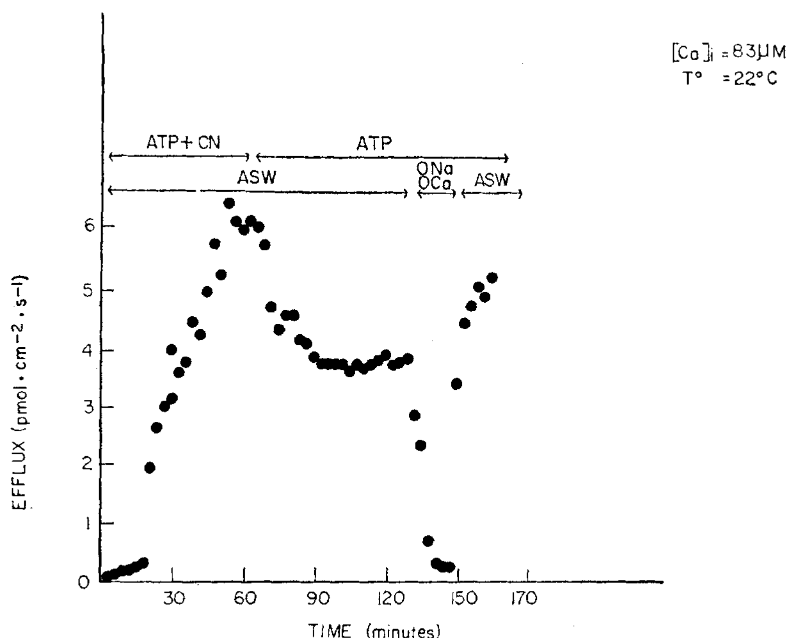

FIGURE 8. The effect of removing the internal cyanide in an axon dialyzed with 5 mM ATP throughout the experiment. The effect of external sodium and calcium was tested by substituting Na^+ with Li^+ and Ca^{++} with Mg^{++}. Abscissa: time in min. Ordinate: calcium efflux in $pmol/cm^2 \cdot s$. Axon diameter 410 μm.

Fig. 8. Removing the cyanide from the dialysis fluid of an axon previously dialyzed with 5 mM ATP + CN^- caused a significant drop in the calcium efflux. The residual efflux is shown to be dependent in a reversible manner on the external sodium and calcium.

DISCUSSION

Rojas and Hidalgo (1968) and Blaustein and Hodgkin (1969) have studied the effect of metabolic inhibitors of the calcium efflux from squid axons. The general conclusion from these experiments is that metabolic poisoning of the cell does not block the calcium efflux, but rather increases it conspicuously, probably due to a release of calcium from the mitochondria. Since in these experiments the level of internal ATP during poisoning does not fall below 100 μM (Caldwell et al., 1960), it is not possible to discard the role of ATP in the sodium-calcium exchange.

The experiments here reported have shown that in dialyzed axons containing ATP concentrations as low as 1 μM and at various $[Ca]_i$ the bulk of the calcium efflux can be primarily explained by an exchange for external sodium and calcium, suggesting that neither of the fractions of the total Ca efflux activated by $[Na]_o$ and $[Ca]_o$ are inhibited in the absence of ATP. This suggests

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that sources of energy other than ATP hydrolysis are involved in driving the bulk of the sodium-calcium counter transport. These experimental findings discard a direct major role for ATP in the sodium-calcium exchange process, however, they do not rule out the possibility of a small ATP-dependent calcium efflux component.[1]

Isolated mitochondria can accumulate massive quantities of calcium by a process depending on electron transport and ATP hydrolysis (Vasington and Murphy, 1962; Rossi and Lehninger, 1963). The dialysis technique applied to intact axons or to extruded axoplasmic samples can give some information on the calcium consumption by the mitochondrial system of the axon. In order to do so, the value of the Ca efflux (when axons were used), calcium concentration in the dialysis effluent, and the degree of porosity of the capillary have to be taken into account.

Under the experimental conditions (Figs. 5, 6) described, the sudden perfusion of 5 mM ATP results in the apparent accumulation of about 3.7×10^{-7} mol Ca^{++}/cm^3 of axoplasm. To ascribe this accumulation to the mitochondrial system, it seems convenient to express it in terms of the mitochondrial proteins of the axoplasm. This allows a comparison of the calcium uptake described in other mitochondrial preparations. The normalized value for the in vivo calcium uptake is in the order of 6.0×10^{-7} mol Ca^{++}/mg of protein, with an initial rate of about 2.6×10^{-8} mol Ca^{++}/min·mg of protein.

Assuming that all the mitochondria in the extruded axoplasm experiment (Fig. 6) contribute to the initial calcium uptake in the presence of 5 mM ATP, it is possible to calculate that the ATP-dependent calcium influx is of the order of 2.6 pmole/cm^2·s. However, this value is most probably an underestimation due to diffusion delays and ATP consumption in the axoplasm. If these factors are taken into account, the initial calcium uptake would reflect mainly the activity of the fraction of mitochondria nearest to the dialysis capillary, and the value given above could increase up to 10-fold. It is necessary to stress that this influx figure refers only to the ATP-dependent fraction of the calcium influx. From the data of this work no estimate can be given for the mitochondrial calcium influx fractions that depend on other energy sources.

The data presented in this paper permit calculations of approximate values for the ratio between internal mitochondrial Ca^{++} and calcium in the axoplasm. Taking the value of 390 μM of Ca^{++} in the axoplasm (Baker, 1972), and assuming that all of it is evenly distributed in the mitochondrial water, then the Ca^{++} concentration in the mitochondria can be calculated to be 56,000 μM in comparison with an axoplasmic Ca^{++} concentration of about 0.3 μm. The concentration ratio for Ca^{++} is thus about $56{,}000/0.3 = 186{,}666$ in the steady state. However, it is not known whether all the bound calcium in the axoplasm is held inside the mitochondria or whether there are other compart-

[1] See Note Added in Proof.

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ments capable of binding calcium. Moreover, it is unlikely that all the internal mitochondrial calcium is free and has the same thermodynamic coefficient as the external calcium.

The bulk of the calcium efflux from squid axons seems to be dependent on external sodium and calcium (Blaustein and Hodgkin, 1969), it is not blocked by poisoning the nerve fiber, but instead, the application of cyanide or dinitrophenol causes a dramatic rise in the calcium efflux (Rojas and Hidalgo, 1968; Blaustein and Hodgkin, 1969).

The experiments described in this study on the effect of cyanide on the calcium efflux confirm those reported by other authors. However, two major points of interest should be mentioned: first, axons dialyzed with 2 mM internal cyanide show a significant rise in the calcium efflux after about 10 min of internal perfusion. This shorter time delay for the CN^- effect on the calcium efflux when compared with about 20 min in Rojas and Hidalgo (1968) experiments and 30–90 min in those of Blaustein and Hodgkin (1969) can be explained on the grounds that as our dialyzed axons had an average diameter of 440 μm while theirs ranged from 700 to 1000 μm, the CN^- in the axoplasm reached much faster a concentration of about 100 μM needed to completely cut off mitochondrial respiration. A second point of interest was the finding that cyanide at internal concentrations of 2 mM was able to substantially increase the calcium efflux in the presence of 5 mM exogenous ATP. This experimental finding suggests that CN^-, per se, seems to liberate a sizable fraction of bound calcium from internal sources, probably the mitochondrial system, even in the presence of normal amounts of ATP.

Another possible explanation for the effect of CN^- on the calcium efflux in the presence of exogenous ATP could reside in a deleterious action of internally applied cyanide at 2 mM on the mitochondrial system. However, the observed decrease in the calcium efflux when CN^- is removed from the perfusion solution (Fig. 8) would not be expected according to this explanation. Whatever the ultimate cause of the CN^- effect, these experiments suggest that the rise in the calcium efflux observed in intact poisoned axons might be the consequence of both a decrease in the internal ATP concentration which has been shown to release endogenous calcium from the mitochondria and a specific CN^- effect on the internally bound calcium.

Note Added in Proof. Recent unpublished experiments in this laboratory have shown that at low $[Ca]_i$ there is an ATP-dependent component of the Ca efflux, possibly suggesting the presence of a "Ca pump."

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