Characterization of the ATP-Dependent Calcium Efflux in Dialyzed Squid Giant Axons

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ABSTRACT The magnitude of the activating effect of ATP on the Ca efflux was explored at different [Ca++] in squid axons previously exposed to cyanide seawater and internally dialyzed with a medium free of ATP and containing p-trifluoro-methoxy carbonyl cyanide phenyl hydrazine. At the lowest [Ca++] used (0.06 μM) more than 95% of the Ca efflux depends on ATP. At high [Ca++] (100 μM), 50-60% of the Ca efflux still depends on ATP. The apparent affinity constant for ATP was not significantly affected in the range of [Ca++] from 0.06 to 1 μM. Axons dialyzed to reduce their internal magnesium failed to show the usual activation of the Ca efflux when the Tris or the sodium salt of ATP was used. Only in the presence of internal magnesium is ATP able to stimulate the Ca efflux. Nine naturally occurring high-energy phosphate compounds were ineffective in supporting calcium efflux. These compounds were: UTP, GTP, CTP, UDP, CDP, ADP, AMP, C-AMP, and acetyl phosphate. The compounds 2’deoxy-ATP and the hydrolyzable analog α,β-methylene ATP were able to activate the Ca efflux. The nonhydrolyzable analog βγ-methylene ATP competes with ATP for the activating site, but is unable to activate the Ca efflux. The results are discussed in terms of the specificity of the nucleotide site responsible for the ATP-dependent Ca efflux.

INTRODUCTION

When squid axons are exposed to seawater of different Na concentrations, an exchange of internal Ca for external Na across the cell membrane may be brought into evidence (Baker, 1972). This exchange, which is thought to represent an effective way of controlling the intracellular ionized calcium concentration, is seen in cyanide-poisoned axons (Blaustein and Hodgkin, 1969) and in the axons dialyzed free of ATP (DiPolo, 1973). It is thought that the energy needed for the extrusion of Ca could be derived from that stored in the sodium electrochemical gradient across the cell membrane. In fact, Blaustein and Hodgkin (1969) suggested that if the stoichiometry of the Na-Ca exchange mechanism were to be three to one, the sodium electrochemical gradient alone would provide sufficient energy to decrease the ionized calcium concentration in the axoplasm to about 0.1 μM. More recently it has been found that the calcium efflux is affected by the intracellular levels of ATP (Baker and Glitsch, 1973; DiPolo, 1973, 1974). Specifically, DiPolo (1974, 1976) has shown that in cyanide-
poisoned squid axons depleted of ATP by prolonged dialysis, the calcium efflux can be substantially increased when ATP is added to the dialysis fluid.

DiPolo et al. (1976), using aequorin and arsenazo as calcium indicators, found that the ionized calcium concentration in fresh intact axons is of the order of 0.02–0.06 μM. Since most of the previous work on Ca efflux and its energetics has been carried out by using either high or undetermined internal calcium concentrations, it was clearly necessary to re-examine the effect of ATP on calcium efflux at internal calcium concentrations near the physiological value.

In the first part of this paper, it is shown that at an ionized calcium concentration of 0.06 μM, the Ca efflux is totally dependent on the presence of internal ATP. Moreover, at this low calcium concentration the remaining flux observed in the absence of ATP does not show the usual dependency on external sodium and calcium and probably can be accounted for by leak of the Ca-EGTA complex through the membrane (Brinley et al., 1975).

Confirming previous results (DiPolo, 1973; Blaustein et al., 1974; Brinley et al., 1975) at higher ionized calcium concentrations, an ATP-independent calcium efflux level is observable.

In view of the existence of an effect of ATP on calcium extrusion, it is of interest to know whether ATP is hydrolyzed or if it only interacts with the transporting mechanism without undergoing catalytic hydrolysis during the translocation of Ca across the membrane. The measurement of inorganic phosphate released, if any, during Ca transport would be the most direct way of answering this question. Unfortunately, this approach presents great experimental difficulties due to the presence in the axoplasm of unspecific ATPases which could mask the activity of any membrane ATPase involved in calcium transport. As an alternative approach in the second part of this paper, we have studied the nucleotide requirement of the transport mechanism, testing the ability of nonhydrolyzable analogs of ATP, hydrolyzable analogs, and other nucleotides in promoting efflux. Third, in order further to characterize the ATP effect, we have explored the possible role of naturally occurring compounds such as ADP and Mg++.

It will be shown that only ATP and its hydrolyzable analogs, 2'deoxy-ATP (d-ATP) and α,β methylene ATP (AMPCP-P), can stimulate the Ca efflux in dialyzed axons. Interestingly, the nonhydrolyzable analog, β,γ methylene-ATP (AMP-PCP) and other nucleotides tested were found to be ineffective as substitutes of ATP. These compounds, on the contrary, seem to compete with ATP for a site in the transport system. Finally, it is reported in this paper that internal magnesium constituted a strict requirement for the stimulating action of ATP.

A preliminary report of part of this work has been presented elsewhere (DiPolo, 1976).

**M A T E R I A L S  A N D  M E T H O D S**

The experiments reported herein were performed with live specimens of *Doryteuthis plei*. After decapitation, the hindmost giant axon was dissected from the mantle in seawater. The mean axon diameter was 430 μm.

**Porosity of Acetate Cellulose Dialysis Capillaries**

The apparatus and procedure used to dialyze isolated squid axons have been extensively
described before (Brinley and Mullins, 1967; DiPolo, 1974). Acetate cellulose capillaries (made by Fabric Research Ltd., Dedham, Mass.) about 145 μm OD, 95 μm ID, kindly supplied by Dr. F. J. Brinley, Jr., were employed. The capillaries were made porous by soaking in NaOH (0.05 M) for 24 h. Since it was observed that the porosity of these plastic capillaries was not always the same, it was thought to be of interest to look into the conditions which might affect their porosity. Fig. 1 (top) shows the experimental arrangement used to measure the porosity of the plastic capillaries. A glass capillary, of ~0.2 cm ID and 5 cm in length, was filled with the dialysis solution containing 45Ca (see Table I). The porous capillary was placed inside the glass capillary with the porous region surrounded by the perfusion fluid. A nonradioactive perfusion medium was allowed to flow through the porous capillary at a constant rate of 1 μl/min. The effluent from the porous capillary was collected every 5 min and its radioactive content measured with a liquid

![Diagram](image)

**FIGURE 1.** Measurements of the porosity of cellulose acetate plastic capillaries under different experimental conditions. The inset shows the experimental arrangement (see text for explanation). Ordinate, counts per minute collected from the plastic capillary. Abscissa, time in minutes. •, Capillary tested immediately after opening in 50 mM NaOH for 24 h. ○, Same capillary dried in air for 90 min before testing. ▲, Capillary opened in 50 mM NaOH for 24 h and stored in distilled water for 10 days before being tested for porosity. □, Capillary tested immediately after opening in 5 mM NaOH for 1 wk. ●, Capillary opened in 50 mM NaOH for 24 h and stored in air for 5 days. The numbers above each curve represent the washout half-time for 45Ca (minutes) calculated for an axon of 600 μm dialyzed with a capillary (95 ID, 145 OD) having a porous region of 1.5 cm.
scintillation counter. Fig. 1 shows typical curves for the steady-state rate of removal of radioactive material observed under different experimental conditions (see legend to Fig. 1). From these types of studies, it was found: (a) that a concentration of 50 mM NaOH is optimum for opening the capillaries (lower NaOH concentrations, even for longer periods of time, do not improve porosity [see Fig. 1]); (b) that soaking for more than 24 h does not render the capillaries more permeable (a period of 20–24 h seems to be optimum); (c) that before its opening, the acetate capillary must be handled very carefully to avoid depositing grease on its wall; (d) that dryness and aging of the porous region markedly decrease its permeability to calcium ions (see Fig. 1); (e) that the effect on porosity of conditions such as dryness and age of the capillary applied mainly to polyvalent ions (such as calcium and ATP—trial experiments with sodium ions showed a smaller dependence on these factors); and (f) that capillaries bathed in distilled water showed a low porosity when perfused with the standard medium, suggesting a surface charge phenomenon associated with the matrix of the porous capillary. Some of these points are illustrated in Fig. 1 in which it is clearly seen that the washout time for radioactive calcium depends on the previous history of the capillary. Since these changes were found to be irreversible, all the experiments reported here were performed with freshly prepared capillaries that were kept in 0.1 M KCl solution before use. (Some of these experiments were performed in the summer of 1975 in the Marine Biological Laboratory, Woods Hole, Mass., in collaboration with Dr. L. Beaugé and Dr. J. Requena).

### TABLE I

| Substance   | ASW* | Internal dialysis |
|-------------|------|-------------------|
|             | mM   | mM                |
| Potassium   | 10   | 320               |
| Sodium      | 442  | 80                |
| Magnesium   | 53   | 5                 |
| Calcium     | 10   | §                 |
| Chloride    | 583  | 90                |
| Aspartate   | —    | 155¶              |
| Isethionate | —    | 155               |
| HEPES       | —    | 10                |
| Tris        | 10   | —                 |
| Glycine     | —    | 160               |

* KCN (1 mM) was always added to the artificial seawater (ASW).

¶ Due to the impurity of isethionate in most cases aspartate alone was used as potassium salt.

**ATP Analysis**

ATP analyses were performed with the firefly flash method (Brinley and Mullins, 1967). Measurements of ATP concentration in the dialysis effluent were made by collecting 5 µl of the perfusate in a glass capillary tube. The samples were measured for ATP content immediately after collection. This procedure permits obtaining the time course of ATP washout from the fiber. In this work, the range of ATP concentrations in the dialysis
effluent, measured from cyanide-poisoned axons extensively dialyzed without high-energy phosphate compounds, was from 8 to 15 μM (no prewash of end regions). However, when the dialysis medium also contained p-trifluoro methoxy carbonyl cyanide phenyl hydrazine (FCCP), a potent uncoupler of mitochondrial metabolism, it was found, first, that the time necessary to deplete the axons of ATP was significantly reduced and second, that no ATP could be detected in the dialysis effluent after a suitable dialysis period (see Table III). Therefore, this compound was employed in most of the experiments reported in this work.

**Solutions**

The solutions used in this study are given in Table I. The osmolarity of all solutions used was determined by using a commercial psychrometer (Wescor Inc., Logan, Utah). External solutions were adjusted to 1,010 mosmol/kg and the internal solutions to 975 mosmol/kg. The calcium contamination in the dialysis solution was routinely checked by atomic absorption spectrophotometry. Since it was found that K-isethionate (Eastman Kodak Corp., Organic Chemicals Div., Rochester, N. Y.) was heavily contaminated with Ca and Na ions, only aspartate was used as potassium salt in most experiments. The ionized Ca concentration in the dialysis fluid was fixed by the use of Ca-EGTA-EGTA buffer system. The total EGTA concentration used ranged from 0.2 to 1 mM. The ionized calcium present in the dialysis medium was calculated with an apparent dissociation constant for the Ca-EGTA complex of 150 nM (DiPolo et al., 1976). In our experiments we normally used Tris as external Na substitute.

Radioactive solutions were made by adding solid ⁴⁰Ca Cl₂ (New England Nuclear, Boston, Mass., 10–20 mCi/mg) directly to the perfusion medium (~1.6 × 10⁸ cpm/cm³). α,β- and α,γ-methylene ATP were purchased from Boehringer Mannhein GmbH. All other nucleotides (UTP, CTP, GTP, UDP, CDP, ATP, ADP, AMP, UMP, and cyclic AMP) and chemicals such as acetyolphosphate, Tris hydroxymethyl aminomethane (Tris), ethylene glycol-bis (β aminoethyl ether) N,N'-tetraacetic acid (EGTA), and oligomycin, were purchased from Sigma Chemical Co., St. Louis, Mo. FCCP samples were kindly supplied by Dr. A. Scarpa and Dr. M. Blaustein. Phosphoarginine was a generous gift from Dr. L. Mullins.

**RESULTS**

**Effect of ATP on the Calcium Efflux at Different [Ca++]ᵢ**

In order to explore the dependency of the ATP effect on the internal ionized calcium, a series of experiments was carried out that used each axon as its own control. Axons bathed in cyanide-artificial seawater (ASW) were dialyzed with an internal solution containing a given ionized calcium concentration, FCCP, and oligomycin. After reaching a steady state in the Ca efflux level, a test concentration of ATP was added to the dialysis medium. Fig. 2 shows the results of two such experiments in which the effect of ATP was tested at three different internal ionized calcium concentrations: 0.06; 0.55; and 100 μM. The upper left portion of the figure shows that at the beginning of the dialysis, the Ca efflux transiently rises, falling to a steady value slightly greater than the background level. This rapid decay of the efflux is probably due to the fast removal of ATP from the axoplasm and is typical of the experiments performed with low concentrations of internal calcium. Upon addition of ATP (2 mM), the efflux
Figure 2. The effect of ATP on the Ca efflux at three different [Ca]; Ordinate, calcium efflux in pmol·cm⁻²·s⁻¹; Abscissa, time in minutes. The upper figures represent the continuation of the same experiment. Notice the change in scale in the ordinate. In the left upper figure, the background level is shown by a dotted line. The lower figure represents a different axon in which the [Ca] was set at 100 μM.
rose to a value of 0.02 P/CS. The absolute need for ATP is observed in this experiment since the removal of this compound from the dialysis medium produced a fast reduction of the efflux to near-background level. The right portion of the upper record shows the continuation of the same experiment in which the internal perfusion medium was changed to one containing 0.55 μM [Ca++] . Under these circumstances, and in the absence of ATP, the Ca efflux rises to a steady value of 0.05 P/CS. Subsequent addition of ATP produced a 10-fold increase in the Ca efflux. In the lower portion of the figure the effect of ATP is tested on an axon dialyzed with a much higher ionized calcium concentration (100 μM) after a temporary rise in the Ca efflux to a steady value of 1.6 P/CS. Addition of ATP increased the efflux to about 4 P/CS.

It is clear that over a wide range of internal ionized calcium concentrations, ATP stimulates the calcium efflux. It is also evident that at the physiological level of ionized calcium, the Ca efflux, in the absence of ATP, is practically negligible since the amount of radioactivity leaving the axon under these conditions is slightly higher than the background level (1.5 times).

Table II summarizes the results of several experiments similar to those described in Fig. 2. It is interesting to notice that in this set of experiments the

\[ P/CS = \text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}. \]
relative magnitude of the ATP-independent fraction of the Ca efflux, observed at high ionized calcium concentrations, is somewhat smaller than the values previously published (DiPolo, 1974). A possible explanation for this quantitative discrepancy may reside in the higher buffering capacity in the present experiments derived from the use of higher EGTA concentrations in the dialysis medium and the use of a very potent metabolic inhibitor (FCCP).

Fig. 3 shows a plot of the fractional effect of ATP on the calcium efflux vs. the internal ionized calcium concentration. At the physiological level of ionized calcium a 24-fold stimulation caused by ATP is observed. Clearly, as the \([\text{Ca}^{++}]_i\) is increased, the fractional stimulating effect of ATP is decreased. One possible explanation for this phenomenon, namely, a change in the affinity of the calcium-transporting mechanism to ATP due to the rise in the \([\text{Ca}^{++}]_i\), was explored in a few experiments. The results obtained in two such experiments are shown in Fig. 4. The upper portion of the figure shows one experiment in which the \([\text{Ca}^{++}]_i\) was set to 0.06 μM. As already mentioned, in the absence of ATP the residual Ca efflux is practically negligible. Addition of 0.05, 0.2, and 2 mM ATP to the dialysis medium causes increasingly higher steady-state values of the Ca efflux. The lower portion of the figure shows a similar experiment carried out with a higher \([\text{Ca}^{++}]_i\) (0.9 μM). Sequential additions of the same concentrations of ATP cause a fractional increment of the Ca efflux similar to those obtained at the lower \([\text{Ca}^{++}]_i\). From these and other similar experiments it can be deduced that the apparent affinity constant for the activating effect of ATP was not significantly affected by increasing the internal Ca by a 15-fold
factor above the physiological level. A $K_m^{\text{ATP}}$ of ~300 μM obtained in this set of experiments is not significantly different from that which could be deduced from previous data (DiPolo, 1974, 1976).

ATP Effect and Internal Magnesium

It is known that internal magnesium is necessary for ion transport to occur in several systems which require ATP for their functioning (Skou, 1957). It has been reported (Brinley et al., 1975) that great variations in the internal magnesium concentration did not appreciably change the level of the calcium efflux in dialyzed squid axons depleted of ATP.

In order to determine whether internal Mg is required by the Ca transport mechanism when it is activated by ATP, axons were bathed in ASW prepared without this cation and dialyzed with a medium free of Mg before being tested for their ATP effect. A typical experiment is shown in Fig. 5. Under conditions of low ionized calcium, no ATP, and no internal magnesium, the Ca efflux is indistinguishable from background. Addition of 1 mM of Tris-ATP did not activate the Ca efflux; however, substitution for Tris-ATP of magnesium ATP in the dialysis medium caused the usual marked stimulation of the Ca efflux. Similar results were obtained in axons dialyzed with high ionized internal calcium concentrations. From these results it can be concluded that the ATP-dependent calcium efflux requires Mg as a cofactor.

The Effect of ATP Analogs on the Ca Efflux

In the second part of this work, the possibility that ATP might undergo catalytic hydrolysis during the outward movement of calcium has been explored. In order to explore it, we have tested the effect of hydrolyzable and nonhydrolyzable ATP analogs on the Ca efflux. Figs. 6 and 7 illustrate two typical experiments in which three ATP analogs were added to CN−-poisoned axons which had been dialyzed for 1–1.5 h with ATP-free media in order to reduce their ATP content. In the experiment in Fig. 6, after the Ca efflux had reached a steady-state value of 0.25 P/CS, the addition of 2′deoxy-ATP to the dialysis medium caused a trebling in the Ca efflux.

Fig. 7 shows an experiment in which the effects of two other ATP analogs, α,β-methylene ATP (AMPCP-P) and β,γ-methylene ATP (AMP-PCP), were sequentially tested on the same axon. After the Ca efflux had reached a steady-state value of 0.18 P/CS in the absence of ATP, the addition of 2 mM and 4 mM of the hydrolyzable analog AMPCP-P caused an increase in the Ca efflux, which could be abolished by the removal of this compound from the dialysis medium. Subsequent addition of the nonhydrolyzable ATP analog AMP-PCP did not increase the steady-state level of Ca efflux. Finally, substitution of this compound for ATP induces the usual marked increase in the Ca efflux. These results clearly demonstrate that only the hydrolyzable ATP analogs, 2′deoxy ATP and AMPCP-P, are capable of stimulating the Ca efflux. Their effectiveness with respect to ATP in stimulating Ca efflux is as follows: ATP > 2′deoxy ATP > AMPCP-P.

Although AMP-PCP does not activate the Ca efflux, Fig. 8 shows that it does
interact with the nucleotide binding site. In fact, addition of ATP in the presence of this nonhydrolyzable analog produces only a modest increase in the Ca efflux. Subsequent removal of this analog from the dialysis medium greatly enhances the Ca efflux.

Effect of ADP, AMP and other Nucleotides on the Ca Efflux

Since ADP is normally present in squid axoplasm in measurable quantities, it is of interest to know, first, whether it could be used as a substitute for ATP in activating the Ca efflux, and second, whether it is needed as a cofactor for the ATP stimulation of the Ca extrusion mechanism.

Fig. 9 shows an experiment in which ADP was added to a poisoned axon extensively dialyzed to remove high-energy phosphate compounds. Addition of 2 mM ADP caused the calcium efflux to increase from 0.3 P/CS to about 0.6 P/CS; this increase was followed by a slow decline of the efflux level.

The interpretation of this type of experiment is complicated by the presence of the adenylate kinase system in the axoplasm. On the basis of the reaction 2ADP = ATP + AMP, which is catalyzed by this enzyme, one may expect that addition of millimolar amounts of ADP to the dialysis medium would produce

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**FIGURE 4.** (opposite) The effect different concentrations of ATP on the Ca efflux at two different internal calcium concentrations. Ordinate, calcium efflux in pmol·cm⁻²·s⁻¹. Abscissa, time in minutes. In both experiments the effect of removing 75% of the external sodium and calcium was tested. (External sodium was substituted by Tris and calcium by magnesium.)
Figure 6. The effect of 2'-deoxy ATP on the Ca efflux from a dialyzed squid axon. The 2'-deoxy ATP concentration used was 1 mM. The [UTP] and [CTP] were 2 mM. Abscissa, time in minutes. Ordinate, calcium efflux in pmol·cm⁻²·s⁻¹. Axon diameter, 440 μm.

Figure 7. The effect of α,β-methylene ATP, β,γ-methylene ATP, and ATP on the Ca efflux in a dialyzed squid axon. Abscissa, time in minutes. Ordinate, calcium efflux in pmol·cm⁻²·s⁻¹. Axon diameter, 450 μm.
**Figure 8.** The inhibitory effect of $\beta\gamma$-methylene ATP on the ATP-dependent Ca efflux. Abscissa, time in minutes. Ordinate, calcium efflux in pmol cm$^{-2}$ s$^{-1}$. Axon diameter, 400 $\mu$m.

**Figure 9.** The effect of ADP on the Ca efflux from a dialyzed squid axon. Abscissa, time in minutes. Ordinate, calcium efflux in pmol cm$^{-2}$ s$^{-1}$. Temperature, 22$^\circ$C. $\blacklozenge$, $\blacklozenge$, Calcium efflux in the absence of ATP in the perfusate. $\bigcirc$, Calcium efflux in the presence of 1 mM MgADP in the perfusate. $\square$, Calcium efflux in the presence of 1 mM MgADP + 20 mM Tris AMP in the perfusate.
an appreciable quantity of ATP in the axoplasm (Mullins and Brinley, 1967). The possibility that the stimulating effect of ADP might be due to the secondary formation of ATP was explored by adding large amounts of AMP (20 mM) to the dialysis medium, thus driving the above reaction to the left. Since the equilibrium constant for the reaction is about 0.5 (Eggleston and Heus, 1952), the amounts of ATP formed under these conditions could be considered negligible when compared to the threshold ATP concentration needed to activate the Ca efflux (Fig. 4, and DiPolo, 1974). Fig. 9 shows that in the presence of a large excess of AMP, the addition of ADP to the dialysis fluid does not induce the stimulation of the Ca efflux observed when ADP is added alone. It should be pointed out that neither AMP nor cyclic AMP was found to have any effect on the Ca efflux.

A further point concerning ADP is whether it constitutes a strict requirement of the calcium extrusion mechanism activated by ATP. The presence of ATPase systems in the axoplasm and some spontaneous hydrolysis of the ATP in the dialysis solution could result in a finite concentration of ADP in the axon. Some experiments were therefore carried out to test the effectiveness of ATP in promoting Ca efflux in the presence of very low levels of ADP. For this, axons were internally dialyzed with solutions containing 20 mM phosphoarginine.

This compound will convert most of the ADP present to ATP through a chemical reaction catalyzed by arginine phosphokinase present in large amounts in the axoplasm (Mullins and Brinley, 1967). Under these conditions, it was found that ATP was still able to produce its usual stimulating effect on the Ca efflux, suggesting that contrary to the case of internal magnesium, ADP is not apparently required for the activation of the Ca efflux by ATP.

In the experiments already described, the ATP effect was tested at a very low ADP:ATP ratio in the axoplasm. Some experiments were, therefore, performed in which a different ratio was tested by adding millimolar amounts of ADP to the dialysis medium. Fig. 10 shows an experiment in which 2 mM ADP was added to an axon being dialyzed with 1 mM ATP. It can be observed that this compound reversibly inhibits a fraction of the ATP-dependent Ca efflux. 2

We have further investigated the molecular specificity of ATP by comparing the effect of other nucleotide triphosphate (UTP, GTP, CTP), diphosphate (UDP, CDP), and monophosphate (UMP, AMP, C-AMP) compounds on the calcium efflux. They were tested as usual in poisoned axons previously dialyzed with solutions containing no ATP. It was found that none of these nucleotides was able to activate the Ca efflux.

Interestingly, when the triphosphate and diphosphate nucleotides were added in millimolar amounts to axons dialyzed with ATP, an inhibitory action similar to that already described for ADP was obtained. This effect may be observed in Fig. 6.

Finally, the effect of acetylphosphate was explored. This compound is known

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2 It may be argued that the fall in the Ca efflux observed in these experiments may be due to calcium binding by ADP. This possibility was ruled out by calculating the change in ionized calcium produced by the addition of ADP (1-3 mM) to a medium containing excess Mg** (4-8 mM), Ca-EGTA (0.5 mM) buffer, and ATP (1 mM). For this, the binding constants for Ca** and Mg** to ADP and ATP, for squid axoplasm, were employed as calculated by Paul de Weer (personal communication).
to form a phosphorylated intermediate with membrane ATPase preparations (Bond et al., 1966). However, even when used at high concentrations (5-10 mM), it was found ineffective in activating the Ca efflux.

**DISCUSSION**

The results presented here together with those previously reported (DiPolo, 1974, 1976) clearly demonstrate that an essential feature of the outward movement of calcium in squid axons is its dependency on ATP. This phenomenon is observed more clearly in dialyzed axons in which a reliable control of both the ionized level of calcium and the concentration of ATP in the axoplasm can be achieved. The ATP requirement of the Ca efflux is critical at concentrations of internal ionized calcium near the physiological level (0.02-0.06 μM). In fact, as may be observed in Table II and Fig. 4, the residual efflux of Ca in the absence of ATP is practically indistinguishable from the radioactivity background level. This flux has been accounted for in terms of a leak of Ca-EGTA through the axolemma (Brinley et al., 1975). A feature of the experiments reported here is a lower residual Ca efflux in the absence of ATP as compared to those reported by Brinley et al. (1975). A possible explanation for this discrepancy may reside in the use of a potent metabolic inhibitor, FCCP, in the experiments reported here and/or a lower leak of the Ca-EGTA complex in *Doryteuthis* as compared to *Loligo*.

![Graph](image-url)
Paradoxically, when the internal Ca is increased above the physiological level, a component of the Ca efflux which is independent of the presence of ATP but dependent on external sodium and calcium, is observable (DiPolo, 1973; Brinley et al., 1975; Blaustein and Russell, 1975). It may be argued that at a higher internal ionized calcium, the affinity of the Ca-transporting system towards ATP could increase in such a way that residual amounts of ATP (micromolar range) observed in the perfusate of cyanide-poisoned dialyzed axons (Mullins and Brinley, 1967) might be sufficient to drive this fraction of the Ca efflux. If this were to be the case, one should observe a shift in the activation curve of Ca efflux by ATP at various concentrations of internal ionized calcium, and/or residual amounts of ATP should have been detected in the dialyzate during the course of the experiments reported herein. However, as shown in Fig. 4, the activation of the Ca efflux by ATP was not significantly affected by changing the $[\text{Ca}^{+}]_{i}$ 15-fold (0.06–0.9 $\mu$M) and, as shown in Table III, axons dialyzed with solutions containing FCCP and cyanide showed no traces of ATP in the perfusate after a suitable dialysis period. This situation contrasts with that of axons poisoned with cyanide alone, in which micromolar amounts of ATP are always present in the dialyzate.

The relative magnitude of the ATP-dependent and ATP-independent fractions of Ca efflux was shown to be a function of the level of internal ionized Ca (Fig. 3 and Table II). The ATP-dependent component of the Ca efflux at 0.06 $\mu$M of the $[\text{Ca}^{+}]_{i}$ accounts for more than 95% of the total efflux obtained. When the internal ionized calcium is increased by a factor of 1,000–100 $\mu$M, the ATP-dependent component of the Ca efflux, although less important, still represents 50–60% of the total efflux value (see Fig. 2), indicating that even at these exceedingly high values of $[\text{Ca}^{+}]_{i}$, the transporting system is capable of being activated by ATP.

The question of whether ATP hydrolysis is involved in the transport of calcium ions in squid axons is a crucial point when dealing with the energetics of the Na-Ca exchange. As has already been mentioned, at present direct measurement of ATP splitting presents numerous difficulties. An alternative approach, which could give some information concerning the mechanism of action of ATP

| Dialysis time (min) | FCCP + CN- Axon ($\mu$M) | CN- Axon ($\mu$M) |
|---------------------|--------------------------|------------------|
| 20                  | >10                      | 50               |
| 40                  | -                        | 20               |
| 55                  | 0.2                      | -                |
| 80                  | -                        | 8                |
| 95                  | <0.1                     | -                |
| 150                 | 0.0                      | 10               |

Axons were preincubated in CN- ASW for 1 h before dialysis. End regions were not predialyzed.
on the Ca transport system, is to look for the nucleotide specificity of the activating site. The actions of the nonhydrolyzable and hydrolyzable ATP analogs are of interest in view of the recent finding by Simons (1975) that the nonhydrolyzable analogs can support K-K exchange in red cells. These nucleotide analogs possess structures closely related to the naturally occurring polyphosphate groupings, but are resistant to chemical cleavage at the point of methylene substitution (Myers, 1963). The $\beta,\gamma$-methylene analog may function as a substrate in reactions where the natural triphosphates are cleaved at the $\alpha,\beta$ position but as inhibitors in reactions in which the $\beta,\gamma$ bond is normally cleaved.

We have tested the ability of hydrolyzable and nonhydrolyzable ATP analogs to stimulate Ca efflux in ATP-free dialyzed axons. Activation of the Ca efflux by the nonhydrolyzable analog would suggest that ATP hydrolysis is not necessary. The experiments presented here show that only the two hydrolyzable ATP analogs, 2'-deoxy ATP and $\alpha,\beta$-methylene ATP can support Ca efflux. The nonhydrolyzable analog $\beta,\gamma$-methylene ATP is not capable of acting as a substrate for calcium transport although it can interact with the ATP binding site. This suggests that slight structural modifications of the ATP molecule at the level of the $\beta$ and $\gamma$ phosphate bond greatly change its capacity for activating the Ca efflux. Another point of interest that can be concluded from the results presented here is that the adenine part of the ATP molecule constitutes a strict requirement in any compound that is to serve as a substrate for calcium transport, since none of the other nucleotides tested (UTP, GTP, CTP) was able to substitute for ATP in promoting Ca efflux. With respect to the inhibitory action of the analog, $\beta,\gamma$-methylene ATP and that discussed in the text for ADP and other nucleotides, it appears that a competition with ATP for a specific site in the transport mechanism is involved. Although this inhibition is not too strong, since millimolar amounts of the polyphosphate compound are required, nevertheless, it suggests that the phosphate moiety of the nucleotide is involved in this competition since the mononucleotides C-AMP, AMP, and UMP had no such inhibitory effect.

It is well established that magnesium ions play an important role in several transporting systems in which high-energy phosphate compounds are involved. In particular, both the (Na + K) ATPase from peripheral nerves and the Ca ATPase from red cells require magnesium ions for their activation (Skou, 1957; Schatzmann and Vincenzi, 1969). With the aid of internal dialysis we have explored the importance of internal Mg for the activation of the Ca efflux by ATP. The present results indicate that like to other transporting systems energized by ATP, the ATP-dependent Ca efflux has a strict requirement for magnesium ions.

Although there is evidence that the energy stored in the sodium gradient could drive the electrogenic exchange between external sodium and internal calcium (Blustein, 1974; Mullins and Brinley, 1975), nevertheless, the data presented here do not rule out the hydrolytic hypothesis and, on the contrary, would suggest that a phosphorylating step may be involved in the activation of the Ca efflux by ATP. It should be pointed out that the involvement of ATP in Ca transport in squid axons comes only from studies on the outward movement of calcium. Clearly, information is needed on calcium influx and its dependency...
on a variety of experimental conditions (ATP, Na+, Na+, Ca++, Ca++) before one can postulate a definite role for ATP in the maintenance of a physiological concentration of ionized calcium. If ATP is involved in the energetics of the Ca transport, it should be possible to show first that ATP is able to promote a net calcium flux against a Ca electrochemical gradient and second, that the Ca transport system can be related to a specific Ca ATPase located at the axon membrane.

In this context, a Na-stimulated, ouabain-insensitive, Mg-dependent ATPase which is activated by Ca ions in the micromolar range has been found in a highly purified membrane fraction of lobster walking leg nerve (Proverbio and DiPolo, unpublished observations).

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