Effect of ATP on the Calcium Efflux in Dialyzed Squid Giant Axons

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ABSTRACT Dialysis perfusion technique makes it possible to control the internal composition of squid giant axons. Calcium efflux has been studied in the presence and in the virtual absence (<5 μM) of ATP. The mean calcium efflux from axons dialyzed with 0.3 μM ionized calcium, $[ATP]_i > 1{,}000$ μM, and bathed in artificial seawater (ASW) was 0.24 ± 0.02 pmol·cm^{-2}·s^{-1} (P/CS) (n = 8) at 22°C. With $[ATP]_i < 5$ μM the mean efflux was 0.11 ± 0.01 P/CS (n = 15). The curve relating calcium efflux to $[ATP]_i$ shows a constant residual calcium efflux in the range of 1–100 μM $[ATP]_i$. An increase of the calcium efflux is observed when $[ATP]_i$ is >100 μM and saturates at $[ATP]_i > 1{,}000$ μM. The magnitude of the ATP-dependent fraction of the calcium efflux varies with external concentrations of Na^+, Ca^{++}, and Mg^{++}. These results suggest that internal ATP changes the affinity of the calcium transport system for external cations.

INTRODUCTION

In several biological preparations Ca extrusion appears to be dependent on the presence of external sodium and calcium (Blaustein and Hodgkin, 1969; Baker, 1970; Cosmos and Harris, 1961; Goodford, 1967; Reuter and Seitz, 1968; Schachter et al., 1970). The identification of the energy source for this extrusion mechanism has drawn the interest of several workers in the field. In a previous work (DiPolo, 1973) it was shown that in dialyzed squid axons there was a significant sodium- and calcium-dependent calcium efflux in the virtual absence of ATP. This paper deals with experiments carried out to further study the role of ATP on the calcium extrusion in squid axons. The main conclusion reached is that, while in the absence of ATP there is a significant sodium- and calcium-dependent calcium efflux, under certain experimental conditions the calcium efflux is enhanced by the addition of ATP. This latter finding was not clearly observed in the previous work (DiPolo, 1973) due to the experimental conditions employed there. As it will be shown in this paper, the use of low ionized calcium obtained by the buffering action of EGTA in the dialyzing medium plus the poisoning of the mito-

chondrial system with oligomycin allows the observation of the stimulation effect of ATP on the calcium efflux in the presence of external sodium and calcium. These findings are in agreement with the results of Baker and Glitsch (1970) who described a drop in the calcium efflux in squid giant axons injected with apyrase. Furthermore, similar results have been obtained in barnacle muscle fibers (DiPolo and Caputo, submitted for publication).[1]

METHODS

The experiments reported here were performed using live specimens of *Dorytheutis plei*. After decapitation, the hindmost giant axon from the stellate ganglion was dissected from the mantle in flowing seawater and carefully cleaned under a dissecting microscope. The mean axon diameter was 420 ± 40 μm (n = 40).

Solutions

The solutions used in this study are given in Table I. Solutions with different amounts of Na, Ca, and Mg were prepared by mixing standard solutions in the proper amounts. The calcium contamination in the dialysis solution, measured by atomic absorption spectrophotometry, ranged between 20 and 40 μM. A calcium buffering system (EGTA) in concentrations between 70 and 100 μM was used to set the ionized calcium in the order of 0.3 μM. The ionized calcium present in the calcium buffering solutions was calculated according to Portzehl et al. (1964). 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 salt of ATP and UTP were purchased from Sigma Chemical Co., St. Louis, Mo., they were dissolved in appropriate amounts of KOH, and the pH adjusted to 7.0. Stock solutions of ATP and UTP were stored at −30°C. KCN, oligomycin, and EGTA were obtained from Sigma Chemical Co., St. Louis, Mo.

Internal Dialysis

The apparatus and procedures used to dialyze isolated squid axons have been extensively described previously (Brinley and Mullins, 1967). The main difference with the previously used procedure was that most of the fiber length was continuously dialyzed. This procedure makes it possible to follow the ATP washout time-course from the beginning of the dialysis (see Fig. 3). Acetate cellulose capillaries about 100 μm in diameter, kindly supplied by Dr. F. J. Brinley, were employed. They were made porous by soaking in NaOH (0.05 M) during 24–48 h. The porosity was always checked by measuring the amount of Ca^{45} crossing the porous capillary wall when a radioactive solution was perfused through it at a rate of 1 μl/min.

Since the acetate cellulose capillaries are extremely flexible, a different procedure was used to introduce them into the axon. The axon was cannulated at both ends with two glass cannulae. An 80-μm glass capillary was introduced along the length of the fiber until its tip protruded out of the distal cannula. The glass capillary was then connected to the porous capillary through a 25-μm platinum iridium wire in-

[1] DiPolo, R., and Caputo. Manuscript submitted to *J. Gen. Physiol.*

TABLE I
SOLUTIONS

Substance	ASW	Ca^{++}-free Tris ASW	Tris ASW	Ca^{++}-,-Mg^{++}-free Tris ASW	Internal dialysis*
Na^+	442	—	—	—	72
$Tris^+$	10	452	452	548	10
K^+	12	12	12	12	335
Mg^{++}	53	53	53	—	8
Ca^{++}	11	—	11	—	...‡
Cl^-	590	590	590	560	98
$Aspartate^-$	—	—	—	—	335
CN^-	2	2	2	2	2

* Used as standard medium free of ATP. Additions made were: ATP 100, 500, 1,000, or 4,000 μM and oligomycin 5 μg/ml. pH:7.2. Sucrose was added to the medium to control the osmolarity.

‡ Tris EGTA was used to buffer the internal calcium to a value of 0.3 μM.

serted into the lumen of the porous capillary. The dialysis capillary was positioned into the axon simply by withdrawing the glass capillary. The internal perfusion was delivered to the porous capillary by a motor-driven syringe at a rate of 1 μl/min. During the experiment the axons were frequently tested for excitability by extracellular recording of the action potential.

ATP Analysis

ATP analyses were performed employing the firefly flash method as modified by Mullins and Brinley (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. All experiments were carried out at room temperature (20–22°C).

RESULTS

Ca Efflux in the Presence of External Na and Ca

In agreement with previous work (DiPolo, 1973), the mean resting calcium efflux in eight squid axons bathed in ASW and dialyzed with solutions containing 0.27 μM free ionized calcium and more than 1 mM ATP was 0.24 ± 0.02 $pmol \cdot cm^{-2} \cdot s^{-1}$ (P/CS). Table II shows that in 15 axons dialyzed with solutions prepared without ATP the mean Ca efflux was 0.11 ± 0.01 P/CS.

Fig. 1 shows the effect of adding ATP on the Ca efflux from an axon bathed in artificial seawater (ASW). Before adding the ATP, the axon was dialyzed with an ATP-free medium and the analysis of the dialysis effluent gave a steady ATP concentration of 2 μM. With this low ATP level, a steady-state Ca efflux value of 0.08 P/CS was observed. The addition of 100 μM ATP to the dialysis medium did not change the efflux level. Yet increasing the [ATP] in the dialysis fluid to 1 mM stimulated the Ca efflux to a value of about

TABLE II

CALCIUM EFFLUXES IN DIALYZED SQUID GIANT AXONS

Fiber reference	$[ATP]_i$	Ca efflux							
		ASW		0 Na, 0 Ca		25% Na, 0 Ca		100% Na, 0 Ca	
		ATP	No ATP	ATP	No ATP	ATP	No ATP	ATP	No ATP
	μM	$pmol \cdot cm^{-2} \cdot s^{-1}$‡		$pmol \cdot cm^{-2} \cdot s^{-1}$		$pmol \cdot cm^{-2} \cdot s^{-1}$		$pmol \cdot cm^{-2} \cdot s^{-1}$	
C20B	3		0.16		0.0047		0.067		0.14
C20A	1,000	0.25							
C21	4,000	0.33		0.011		0.21			
C22	2		0.16		0.0067		0.043		
C23	2		0.10						
C24	3		0.13						
C25	1		0.10						
C25A	100	0.11							
C26	3		0.08						
C26A	1,000	0.27		0.02		0.14			
C2	3		0.16		0.008		0.045		
C2A	4,000			0.04		0.10			
C4	4		0.16		0.008				
C4A	4,000	0.26		0.03					
C5	1		0.15		0.0035		0.06		
C27	2		0.07						
C27A	500	0.14							
C27B	1,000	0.18							
C27C	4,000	0.20		0.02		0.10		0.17	
C28	2		0.051						
C28A	100	0.051							
C28B	500	0.18							
C30	3								
C30A	1,000	0.20							
C30B	100	0.08							
C3	2		0.08						
C31	4		0.09		0.008		0.02		0.076
C31A	4,000	0.25		0.03		0.14		0.21	
C31B	100	0.09							
C32	2		0.09		0.002		0.03		0.08
C32A	4,000			0.02		0.12		0.18	
	Mean ± SEM	0.24 ± 0.02* (n = 8)	0.11 ± 0.01 (n = 15)	0.02 ± 0.004 (n = 7)	0.006 ± 0.001 (n = 7)	0.14 ± 0.02 (n = 6)	0.04 ± 0.007 (n = 6)	0.18 ± 0.01 (n = 3)	0.09 ± 0.02 (n = 3)

* Refers to values obtained with $[ATP]_i$ greater than 1,000 μM.

‡ Referred to as P/CS in text.

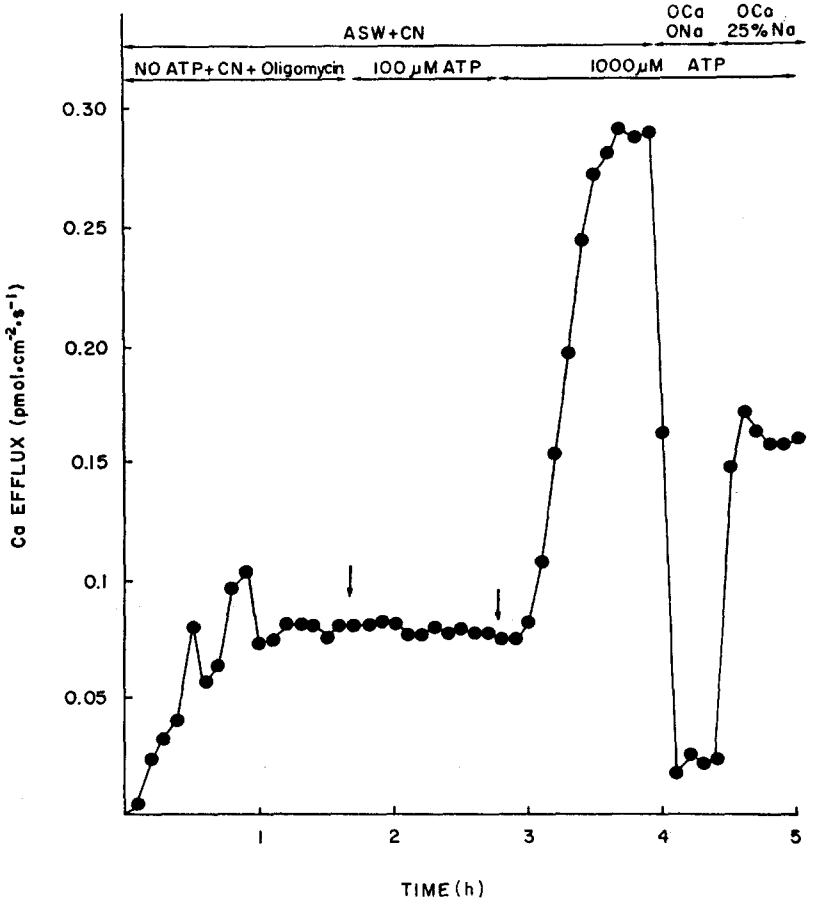

FIGURE 1. The effect of increasing concentrations of ATP on the calcium efflux in a dialyzed squid axon. The ATP values refer to the [ATP] in the fluid entering the dialysis capillary. Abscissa: time in hours. Ordinate: calcium efflux in pmol·cm^{-2}·s^{-1} (P/CS in text). The effect of external sodium and calcium are tested during the course of the experiment. $[Ca]_i$ = 0.28 μM, $T°$: 22°C. Axon diameter: 430 μM.

0.28 P/CS. Under this condition the removal of the external calcium and sodium dropped the efflux to 0.02 P/CS showing that most of the calcium efflux is Na and Ca dependent.

Fig. 2 shows the relationship between the Ca efflux and the internal ATP concentration as obtained from various experiments similar to the one described above (see Table II). Two interesting features are worth pointing out: First, when the $[ATP]_i$ is lower than 100 μM, a substantial fraction of the normal calcium efflux is observed. As shown before (DiPolo, 1973) and confirmed here, this fraction is dependent on the presence of external sodium and calcium. Second, the stimulating effect of ATP on the Ca efflux takes

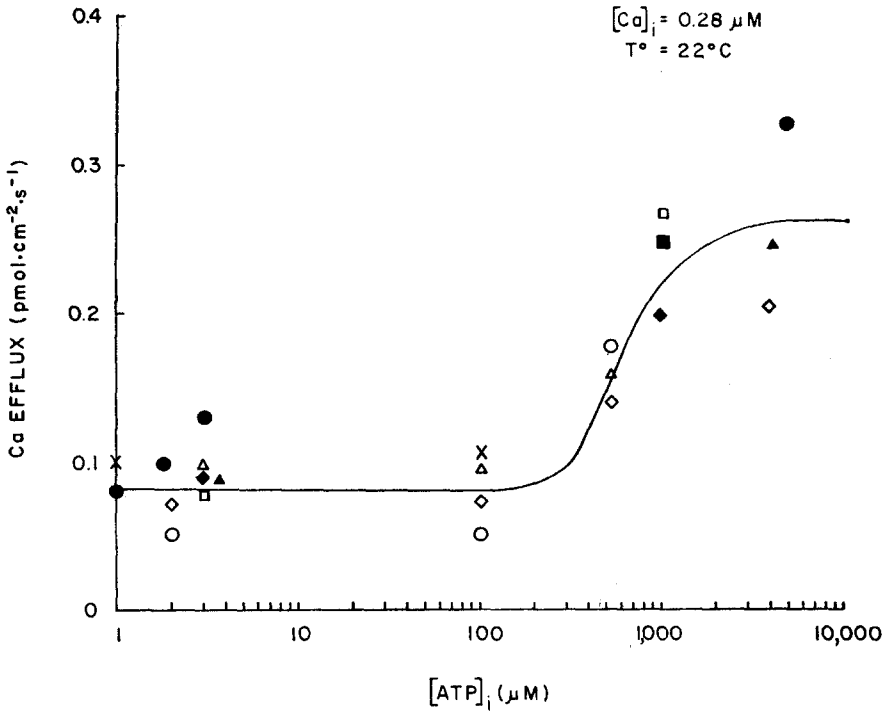

FIGURE 2. The relationship between $[ATP]_i$ and Ca efflux. ● represents four single values from individual axons, while the other symbols refer to axons in which more than one [ATP] value was tested during dialysis. Abscissa: $[ATP]_i$ in a logarithmic scale. Oridinate: calcium efflux in pmol·cm^{-2}·s^{-1}.

place mostly in the concentration range of 100–1,000 μM ATP. A further increment in the ATP concentration does not appear to enhance the calcium efflux.

Effectiveness of Dialysis in Lowering the Intracellular ATP

As shown in Fig. 2 and Table II, a substantial fraction of the calcium efflux remains even when analysis of ATP in the dialysis effluent yields values of less than 5 μM. Obviously, the question arises as to whether this measured ATP represents the concentration of [ATP] at the membrane level. It is conceivable that an ATP concentration gradient could exist between the nerve membrane and the wall of the porous capillary such that a high ATP concentration near the nerve membrane could be responsible for the residual fraction of the Ca efflux observed. Although very unlikely, the importance of this point requires an independent test of the effectiveness of the dialysis technique in reducing the concentration of ATP at the membrane level to that of the dialyzed effluent. It is known that the ATP-sensitive Na efflux in squid axons is almost maximally activated in the range of concentrations

1–100 μM ATP (Brinley and Mullins, 1968). This dependence can therefore be utilized as a criterion of the adequacy of the dialysis technique under the experimental conditions of this work.

Fig. 3 shows the time-course of the sodium efflux in an axon in which dialysis was started with an ATP-free solution. It can be observed at the beginning of the dialysis that the sodium efflux transiently reached a peak value of ≃30 P/CS decreasing later to a steady level of ≃6 P/CS. It can be

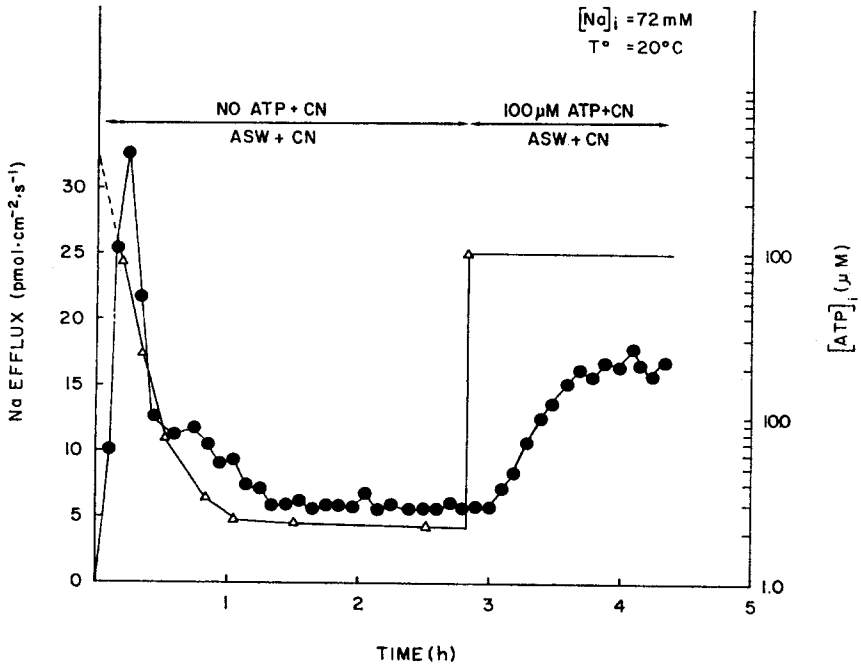

FIGURE 3. The effect of $[ATP]_i$ on the Na efflux in a dialyzed squid axon. ● refers to Na efflux in pmol·cm^{-2}·s^{-1}. △ represents the $[ATP]_i$ in the effluent dialysate. Abscissa: time in hours. At $t = 0$, the dialysis was started with a medium containing Na^{22} and no ATP. The vertical arrow indicates addition of 100 μM ATP to the dialysis medium.

seen that the fall in the efflux follows the reduction of the concentration of ATP in the dialysis effluent. A subsequent addition of 100 μM ATP to the dialysis medium caused an increase in the Na efflux to a value of ≃16.5 P/CS. The transient peak in the Na efflux can be attributed to the fact that radioactive sodium reaches the membrane at the time when the ATP level near the membrane has not yet been appreciably reduced. These results agree with those reported by Brinley and Mullins (1968) and offer additional evidence of the effectiveness of dialysis in lowering [ATP] in the vicinity of the membrane to less than 10 μM when there is no ATP present in the dialysis fluid.

A transient peak in the Ca efflux at the beginning of the dialysis was not seen in the experiment reported in Fig. 1, although it was observed in other experiments such as that of Fig. 8. The slower time-course for radioactive calcium to reach a steady concentration at the membrane level could explain this phenomena. Experimental conditions, such as capillary porosity and axon diameter, could be the determining factor for the appearance of the transient efflux peak.

Ineffectiveness of UTP in Stimulating the Ca Efflux

Fig. 4 shows an experiment designed to test whether UTP, another high energy phosphate compound, could also stimulate the Ca efflux. It can be seen that addition of 1 mM UTP did not change the Ca efflux, while subsequent addition of 0.5 mM ATP raised the calcium efflux from 0.08 to 0.18 P/CS. The same UTP concentration was tested in two other fibers with the same result. This suggests that whatever the mechanism of ATP stimulation may be, it is rather specific. It is interesting to point out that Brinley and Mullins (1968) have obtained similar results regarding the specificity of the sodium pump to high energy phosphate compounds in dialyzed squid axons.

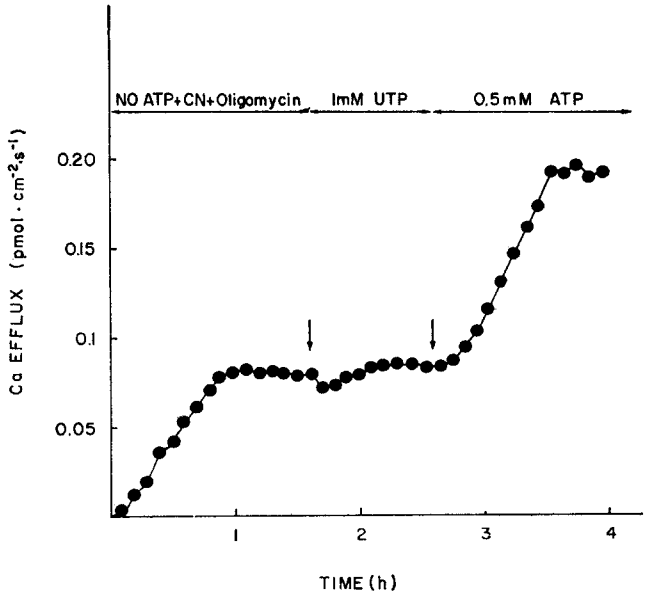

FIGURE 4. The effect of UTP on the Ca efflux. Abscissa: time in hours. Ordinate: Ca efflux in pmol·cm^{-2}·s^{-1}. The first and second vertical arrows indicate addition of 1 mM UTP and 0.5 mM ATP, respectively. $[Ca]_i$ = 0.28 μM, $T°$ = 22°C. Axon diameter: 410 μM.

Effect of ATP and External Ionic Composition on the Ca Efflux

In the experiments described above, the stimulating effect of ATP on the Ca efflux was observed in axons bathed in normal ASW. In the following experiments the influence of the ionic composition of the external medium on the ATP-dependent calcium efflux is analyzed.

In the experiment of Fig. 5, the removal of external calcium and sodium in the absence of ATP in the dialysis medium causes the Ca efflux to fall from a steady value of 0.09 P/CS to 0.002 P/CS. Restoration to 25% and later on to normal external sodium (in the absence of external calcium) causes the calcium efflux to increase to 0.03 and 0.08 P/CS, respectively. Under the last condition, addition of 4 mM ATP to the dialysis medium produces a further increment of Ca efflux to a steady level of 0.18 P/CS. With ATP

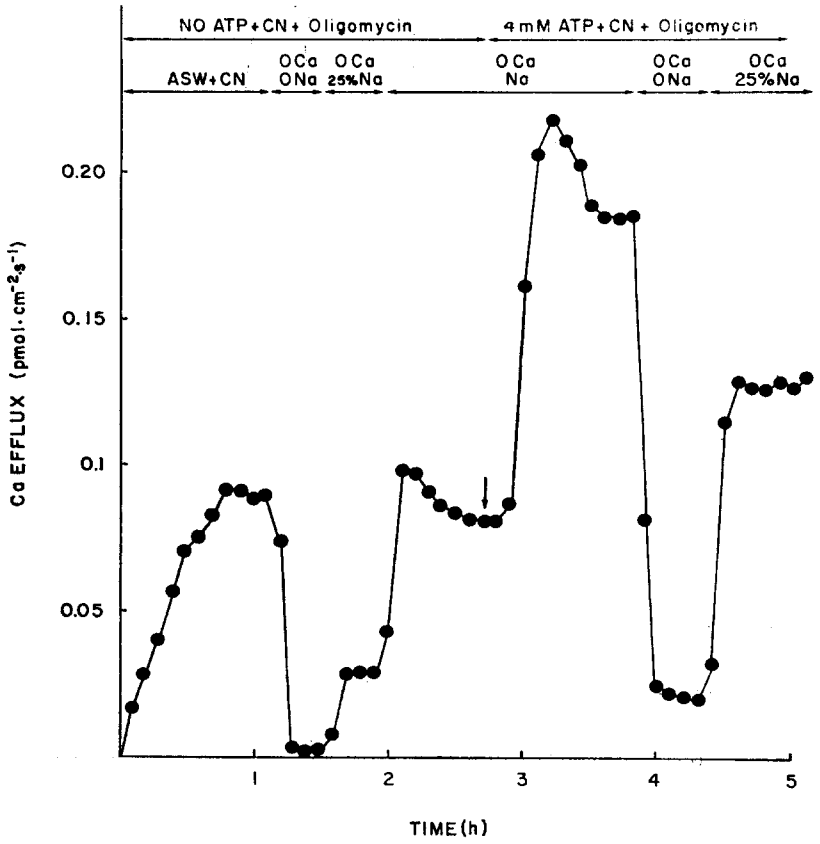

FIGURE 5. The effect of external sodium and ATP on the Ca efflux from a dialyzed squid axon. Abscissa: time in hours. Ordinate: Ca efflux in pmol·cm^{-2}·s^{-1}. The vertical arrow indicates addition of 4 mM ATP to the dialysis medium. $[Ca]_i$ = 0.3 μM; $T°$ = 22°C.

present, removal of external sodium causes the efflux to fall to ≃0.02 P/CS. Finally, restoration of 25% external sodium brings the efflux to about 0.12 P/CS. Fig. 6 shows similar runs carried out with two different axons, one dialyzed with and the other without ATP in which the effect of 25% external calcium was tested in the absence of external sodium. A small stimulating effect was observed which was greater in the axon dialyzed with ATP.

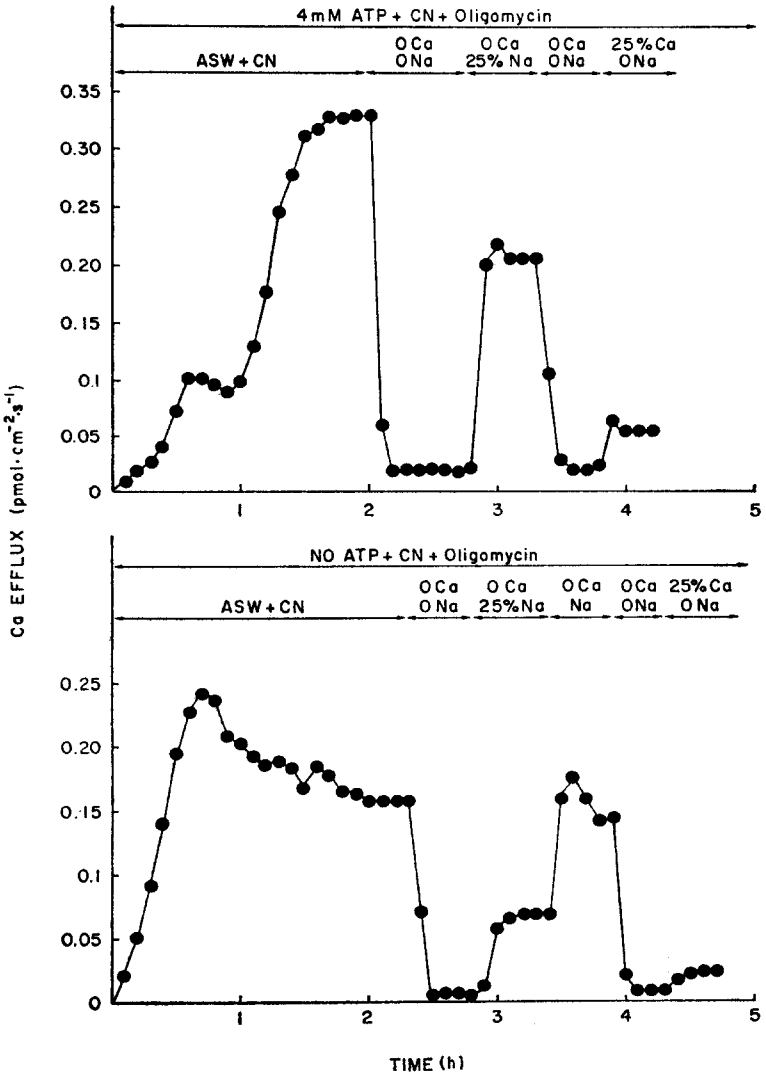

FIGURE 6. The effect of external sodium and calcium on the Ca efflux from a dialyzed squid axon. The upper portion of the figure was obtained with an axon dialyzed with 4 mM ATP in the dialysis medium. The lower portion of the figure was obtained with no ATP in the dialysis fluid. Abscissa: time in hours. Ordinate: Ca efflux in pmol·cm^{-2}·s^{-1}. $(Ca]_i$ = 0.3 μM. $T°$ = 22°C.

Fig. 7 summarizes the results of several experiments in which the effect on the calcium efflux of external sodium, in the absence of external calcium, was tested on axons dialyzed with and without ATP. It is evident that ATP increases the sensitivity of the calcium transport system to external sodium.

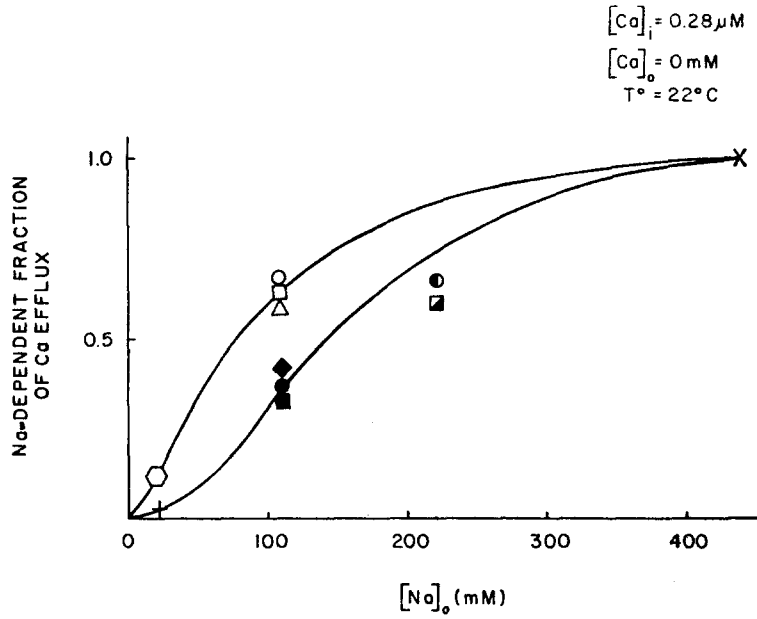

FIGURE 7. The relationship between the Na-dependent fraction of the calcium efflux and the external sodium concentration. The external medium contains no calcium. The different symbols represent values obtained with different axons. The hollow symbol refer to values obtained in the presence of ATP (>1,000 μM). The others to values obtained in the absence of ATP. The curves were fitted using an equation of the type.

$$\frac{Y}{Y_{max}} = \frac{1}{\frac{K}{[Na]_o^n} + 1},$$

where Y is the calcium efflux at any given $[Na]_o$ and Y_{max} is the calcium efflux at 100% external sodium. $K_{1/2}^{ATP}$ = 80 mM, n = 1.5, $K_{1/2}^{OATP}$ = 144 mM, n = 2.

As shown in Figs. 5 and 6 and Table II in the absence of external calcium and sodium, the Ca efflux is consistently greater when ATP is present in the dialysis medium. This is also seen in Fig. 8, and used to show that the stimulating effect of ATP under these conditions can be reversed by removal of the external Mg^{++}. This magnesium-dependent flux is mostly visible in the presence of ATP. However, it can also be visualized in its absence, as shown in Fig. 9, in which the relative magnitudes of the magnesium-, calcium-, and sodium-dependent fractions of the calcium efflux are shown.

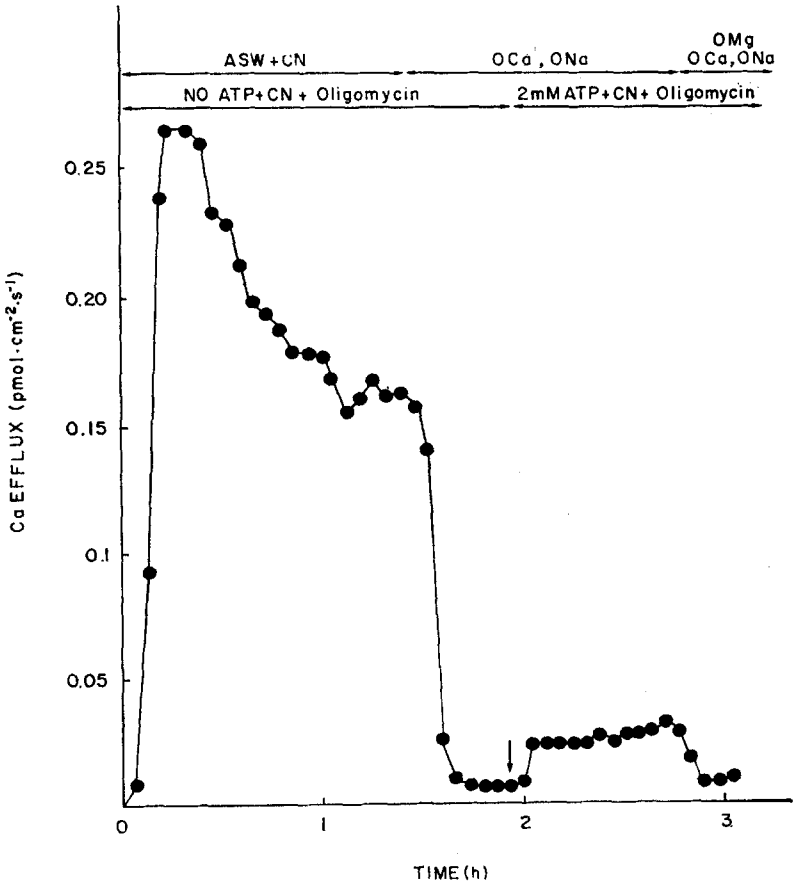

FIGURE 8. The effect of external magnesium on the fraction of the calcium efflux stimulated by ATP in the absence of external sodium and calcium. Abscissa: time in hours. Ordinate: calcium efflux in pmol·cm^{-2}·s^{-1}. $[Ca]_i$ = 0.3 μM. $T°$ = 22°C. Axon diameter: 470 μm.

DISCUSSION

The results presented in this work show that in addition to an ATP-independent fraction of the Ca efflux (DiPolo, 1973) there is a component which is stimulated by relatively high ATP concentrations. This finding agrees with the work of DiPolo and Caputo (submitted for publication) which describes the stimulation of the Ca efflux in barnacle muscle fibers by ATP and with the previous report of Baker and Glitsch (1973) who observed an inhibition of the Ca efflux in squid axon treated with apyrase known to be a powerful ATP hydrolyzing agent. Carefully controlled experimental conditions must be met in order to visualize the ATP-induced stimulation of the Ca efflux otherwise the opposite effect can be observed. For instance, DiPolo (1973)

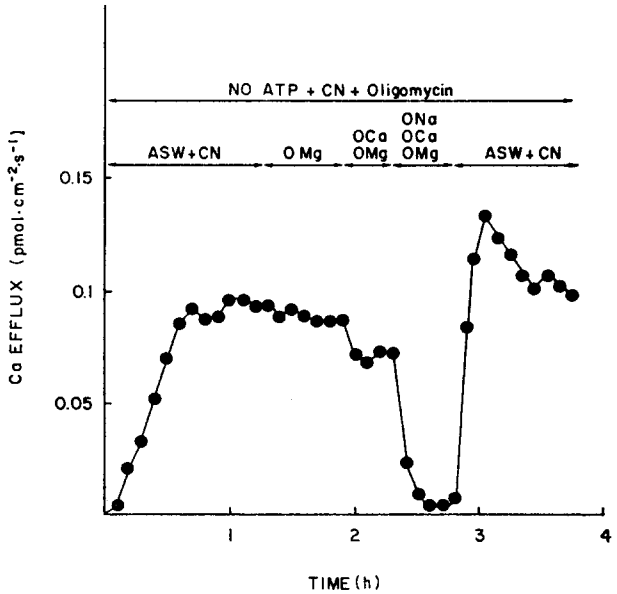

FIGURE 9. The effect of external magnesium, calcium, and sodium on the calcium efflux in the absence of internal ATP. Abscissa: time in hours. Ordinate: calcium efflux in pmol·cm^{-2}·s^{-1}. $[Ca]_i$ = 0.3 μM. $T°$ = 22°C. Axon diameter: 400 μm.

has shown that addition of ATP to axons dialyzed with a medium containing no calcium buffer (80 μM free calcium) and no oligomycin causes a transient fall in the Ca efflux. This fall was explained in terms of calcium uptake by the mitochondrial system. In the present work, this finding was minimized by the use of an internal calcium buffer and by poisoning the mitochondrial system with oligomycin.

It is also shown that the magnitude of the effect of internal ATP on the Ca efflux depends on the composition of the external medium. Axons bathed in normal ASW with no ATP in the dialysis fluid showed a Ca efflux which was 46% of that obtained when ATP (>1 mM) was present. Addition of ATP to axons bathed in ASW media prepared without calcium and sodium increased the calcium efflux from a negligible value to $\simeq$8% of that obtained in normal ASW. This efflux is dependent on the presence of external Mg^{++} and can also be observed when no ATP is present in the internal medium. Its magnitude however is smaller than when ATP is present, suggesting some sort of potentiation effect of ATP on a preexisting mechanism.

A plausible explanation of these results is that there is only one exchange mechanism whose affinity for external cations is modified by the presence of internal ATP. An alternative explanation is to consider the ATP-insensitive and ATP-sensitive fractions of the total calcium efflux as two distinct components of two different transport mechanisms: one which allows exchange

of the internal calcium for external sodium, calcium, and magnesium which does not require metabolic energy, and the other one which does require it. The experimental data obtained in the present work do not give much support to the latter hypothesis. In fact, both fractions of the Ca efflux show the same qualitative dependence on the ionic composition of the external medium, thus making it difficult to sustain the idea of two different components. Furthermore, the ATP requirements of the Ca exchange far exceed those normally necessary in other transport-ATP-energized mechanisms. The relationship between the ATP-sensitive fraction of the Ca efflux and the $[ATP]_i$ can be described by a curve having a half saturation value of 543 μM. If a comparison is made with a similar curve obtained for the sodium efflux in the same preparation (Brinley and Mullins, 1968), the difference in the ATP requirement is striking since the Na efflux is activated by $\simeq$10 μM internal ATP. Nevertheless, these arguments do not completely rule out the possibility of the existence of a metabolically driven fraction of the Ca efflux. Additional evidence, such as the relationship between Ca transport and ATP hydrolysis, is needed to postulate on firmer grounds the existence of a "calcium pump."

Considering the evidence available at present, it seems possible that ATP changes the affinity of the mechanism for the calcium transport in the presence of external sodium as shown in Fig. 7. This explanation agrees with the interpretation that Baker and Glitsch (1973) gave to their experimental results. It is not necessary to postulate that ATP hydrolysis is involved in the mechanism responsible for the affinity change. The case of the Na:Na exchange in red cells (Garrahan and Glynn, 1967) can be used as an example for a mechanism of this kind. In this preparation, ATP is required to maintain a Na:Na exchange, yet little or no hydrolysis of this compound occurs.

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