Biochimica et Biophysica Acta 860 (1986) 583–591
Elsevier

BBA 73248

ATP-dependent calcium pump and Na^+-Ca^{2+} exchange in plasma membrane vesicles from squid optic nerve

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(Received March 28th, 1986)

Key words: Ca^{2+} transport; Na^+-Ca^{2+} exchange; Kinetics; (Squid optic nerve)

Purified plasma membrane vesicles from the optic nerve of the squid *Sepiotheutis sepioidea* accumulate calcium in the presence of Mg^{2+} and ATP. Addition of the Ca^{2+} ionophore A23187 to vesicles which have reached a steady state of calcium-active uptake induces complete discharge of the accumulated cation. Kinetic analysis of the data indicates that the apparent K_m for free Ca^{2+} and ATP are 0.2 μM and 21 μM, respectively. The average V_{max} is 1 nmol Ca^{2+}/min per mg protein at 25°C. This active transport is inhibited by orthovanadate in the micromolar range. An Na^+-Ca^{2+} exchange mechanism is also present in the squid optic nerve membrane. When an outwardly directed Na^+ gradient is imposed on the vesicles, they accumulate calcium in the absence of Mg^{2+} and/or ATP. This ability to accumulate Ca^{2+} is absolutely dependent on the Na^+ gradient: replacement of Na^+ by K^+, or passive dissipation of the Na^+ gradient, abolishes transport activity. The apparent K_m for Ca^{2+} of the Na^+-Ca^{2+} exchange is more than 10-fold higher than that of the ATP-driven pump (app. K_m = 7.5 μM). While the apparent K_m for Na^+ is 74 mM, the V_{max} of the exchanger is 27 nmol Ca^{2+}/min per mg protein at 25°C. These characteristics are comparable to those displayed by the uncoupled Ca pump and Na^+-Ca^{2+} exchange previously described in dialyzed squid axons.

Introduction

Two plasma membrane transport systems, the ATP-dependent Ca^{2+} pump and the Na^+-Ca^{2+} exchanger, have been shown to play a crucial role in the regulation of cytosolic free calcium in a variety of cells [1–7]. The relative contribution of these mechanisms to intracellular calcium homeostasis still remains to be clearly established. In squid axons, it has been proposed that the uncoupled Ca^{2+} pump is responsible for maintaining low resting Ca^{2+} levels, while Na^+-Ca^{2+} exchange handles the large amounts of Ca^{2+} entering the axons during electrical stimulation [8,9].

Recently, a purified plasma membrane preparation has been obtained from squid optic nerves. This preparation offers a means of biochemical investigation which can parallel electrophysiological studies of these transport systems in squid axons, which had been used as a model for the study of Ca transport [1,2,7]. This plasma membrane preparation contains a (Ca^{2+} + Mg^{2+})-ATPase whose kinetic properties resemble those of the active calcium pump [10,11]. It was therefore of interest to establish whether the same plasma membrane preparation would also contain an ATP-dependent Ca^{2+} transport activity. Further,

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; DMSO, dimethylsulfoxide; HEEDTA, *N*-hydroxyethylenediaminetriacetic acid; NMG, *N*-methyl-D-glucamine.

it might be expected that a Na^+-Ca^{2+} exchange activity, dependent of the presence of a Na^+ gradient, could operate in parallel with the active pump.

In this paper we describe both ATP-dependent Ca^{2+} transport and $Na^+ + Ca^{2+}$ exchange activity in squid optic nerve membrane vesicles. A preliminary report of these findings has been presented elsewhere [12].

Materials and Methods

Membrane preparation. Plasma membrane vesicles were prepared from the optic nerves of the squid *Sepiotheutis sepioidea* as previously described [11]. At the end of the isolation procedure the preparation was suspended in 0.3 M sucrose/10 mM Tris-HCl (pH 7.4), rapidly frozen in solid CO_2 and acetone and stored at −70°C at a final protein concentration of about 5 mg/ml. Under these conditions, both $(Ca^{2+} + Mg^{2+})$-ATPase activity and the transport characteristics of the vesicles were constant over at least 3 months. The average diameter of the membrane vesicles was 0.6 μm, as determined by electron microscopy (negative staining).

Vesicle volume determination. The internal volume of the plasma membrane vesicles was determined by a modification of the procedure described by Padan et al. [13]. Vesicles were thawed at room temperature, diluted 15-fold with 300 mM K-Mops (pH 7.3) (25°C) and centrifuged at 150000 × *g* for 30 min. The pellet was then resuspended in a minimal volume of the same solution.

The distribution of [^{14}C]inulin was used to measure the extravesicular volume, and of ^3H_2O to determine the total volume. 1 ml of a solution containing (in mM) 100 KCl, 3 inulin ([^{14}C]inulin, 1 μCi and ^3H_2O, 2.5 μCi), 200 K-Mops (pH 7.3), and 1 mg protein was incubated at 25°C for 30 min. At the end of the incubation period the tubes were centrifuged at 12000 × *g* for 15 min. The pellet was dissolved in 1% SDS and a small volume was counted simultaneously with a sample of the original incubation medium. The average internal volume obtained from four different preparations, each in quadruplicate, was 1.7 ± 0.14 μl/mg of protein.

$(Na^+ + K^+)$-ATPase assays and sidedness estimation. The total $(Na^+ + K^+)$-ATPase activity was measured as previously reported [11] after a 15 min preincubation of the membranes (1 mg of protein/ml) at 25°C in 0.32 M sucrose/15 mM Tris-HCl (pH 7.3), in the presence or not of 0.2% deoxycholate (0.4 mg deoxycholate/mg of protein). This suspension was then diluted 48-fold with the above sucrose solution, 100 μl were added to the ATPase reaction mixture and incubated for

TABLE I

ESTIMATION OF THE ORIENTATION OF SQUID OPTIC NERVE VESICLES USING THE ASYMMETRIC PROPERTIES OF THE $(Na^+ + K^+)$-ATPase

L, leaky vesicles; IO, inside-out vesicles; RO, right-side-out vesicles.

Assay conditions		ATPase (μmol P_i/mg protein per h)		Subpopulation of vesicles activated	Fraction of total population (%)
Deoxycholate preincubation	addition to assay medium	Total	$(Na^+ + K^+)$-ATPase		
–	–	49.0 (1)			
			20.5 (1 – 2)	L	23
–	1 mM ouabain	28.5 (2)			
–	5 μM monensin	50.1 (3)			
			29.6 (3 – 4)	L + IO	33
–	5 μM monensin + 10 μM digitoxigenin	20.5 (4)			
+	–	99.7 (5)			
			90.3 (6 – 5)	L + IO + RO	100
+	1 mM ouabain	9.4 (6)			

10 min at 25°C. The liberated phosphate was quantified as previously reported [14]. The sidedness of the optic nerve membrane preparation was estimated following the method used by Caroni and Carafoli for sarcolemma vesicles [15]. This procedure reveals that our preparation consists of 23% leaky vesicles, 10% inside-out vesicles and 67% right-side-out vesicles (Table I).

Ca^{2+} pump measurements. The ATP-dependent calcium uptake was measured in the following medium (in mM): 0.5 or 1.0 $MgCl_2$; 0.1 ouabain; 5.0 sodium azide; 0.5 EGTA-Tris; $CaCl_2$ variable ($^{45}CaCl_2$ sp. act. 10^4 cpm/nmol) to give [Ca^{2+}] between 10^{-8} and 10^{-3} M; 100 KCl; 200 K-Mops (pH 7.3 at 25°C). Membrane vesicles at a final concentration of 25–40 μg/100 μl were preincubated in the medium for 5 min at 25°C. The uptake of Ca was initiated by addition of ATP (final concentration indicated in the figure legends) and was rapidly terminated by filtration of 0.1 ml aliquots through Milliopre filters (0.45 μm). The filters were washed twice with 3 ml of an ice-cold solution containing 250 mM KCl/50 mM K-Mops, (pH 7.3), dried and dissolved in scintillation liquid and the retained radioactivity was counted. Free Ca^{2+} concentrations were calculated using the equilibria between Ca^{2+}, Mg^{2+}, EGTA and ATP. The dissociation constants used in computation were (high ionic strength): Mg · ATP 0.7 mM; (De Weer, unpublished results) Mg · EGTA, 30 mM [16]. Ca · ATP 1.4 mM [10], Ca · EGTA 0.00017 mM [17]. In order to minimize the effect of additional calcium from contaminating reagents (10 μM in most experiments) the buffered Ca solutions contained high EGTA concentrations.

Ca^{2+}-sensitive microelectrodes. The preparation and calibration procedure of the neutral ligand Ca^{2+}-sensitive electrodes was similar to that described by DiPolo et al. [17]. Ca uptake was determined in 0.1 ml of a solution containing (in mM) 1.5 $MgCl_2$, 0.1 ouabain, 5 sodium azide, 0.1 $CaCl_2$, 100 KCl, 200 K-Mops (pH 7.3) at room temperature (19°C) and an ATP-regenerating system composed of 0.5 mM phospho*enol*pyruvate and 1 U/ml pyruvate kinase. Membrane vesicles (1 mg/ml) were preincubated in the reaction medium for 5 min. The reaction was initiated by adding Tris-ATP (pH 7.3) to achieve a final concentration of 0.5 mM. The continuous disappearance of the external calcium was measured with the Ca-selective microelectrode and displayed by a pen recorder. When the Ca ionophore A23187 was required, it was dissolved either in ethanol or in distilled dimethylsulfoxide (DMSO). Under these conditions, ethanol or DMSO (less than 0.2%) had no effect per se on the calcium transport.

Na^+-Ca^{2+} exchange measurements. Membrane vesicles (5–7 mg protein/ml) were pre-equilibrated for 30 min at 37°C with Na-Mops or K-Mops. The uptake was initiated by diluting (20–30-fold) 5 μl of pre-equilibrated vesicles in an appropriate external medium containing 100 μM Ca and NMG instead of Na^+ and/or K^+. Additional details appear in the figure legends. Ca uptake was carried out in the absence of ATP. The reaction was terminated by filtration on Millipore filters as indicated before. In order to maintain the free Ca^{2+} concentration fixed in the range 10^{-7}–10^{-3} M, three Ca^{2+} buffers were used: EGTA, HEEDTA and nitrilotriacetic acid. Their apparent dissociation constants (high ionic strength, pH 7.3, 25°C) were: 0.17, 5.4 and 209 μM, respectively [17]. Basal Ca binding (measured in potassium-loaded vesicles) was subtracted from the total uptake.

Reagents. Vanadium-free ATP (Tris salt), EGTA, EDTA, Mops, nitrilotriacetic acid, *N*-methyl D-glucamine, HEEDTA, sodium deoxycholate, Phospho*enol*pyruvate, pyruvate kinase, monensin, valinomycin, digitoxigenin and inulin were purchased from Sigma Chemical Co. $^{45}CaCl_2$ ^3H_2O and [^{14}C]inulin were obtained from New England Nuclear. The ionophore A23187 was obtained from Calbiochem Laboratories. Filters were from Millipore Co. All other reagents were of analytical grade.

Results

ATP-dependent Ca uptake

The time-course of ATP-driven Ca^{2+} uptake into squid optic nerve vesicles is shown in Fig. 1. In the presence of both Mg^{2+} and ATP there was a rapid accumulation of Ca^{2+}, which was linear over the first 5 min and reached a maximum level of about 8 nmol/mg of protein in 10 min. Addition of calcium ionophore A23187 after steady

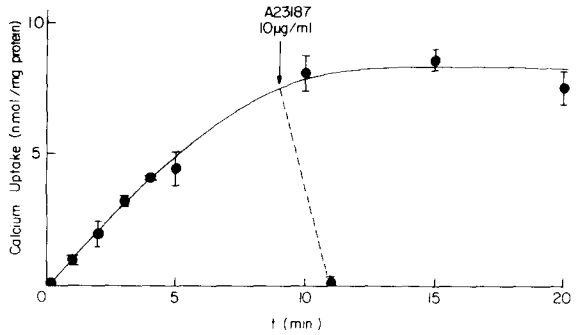

Fig. 1. Time-course of ATP-dependent calcium uptake in optic nerve plasma membrane vesicles. Vesicles (0.3 mg protein/ml) were preincubated 5 min at 25°C in a medium containing (in mM): 0.5 $MgCl_2$, 0.1 ouabain, 5 sodium azide, 0.5 EGTA-Tris, 0.491 $^{45}CaCl_2$ (^{45}Ca sp. act. 10^5 cpm/nmol); 100 KCl, 200 K-Mops (pH 7.3). Under these conditions free Ca^{2+} concentration was 4 μM. Calcium uptake was initiated by addition of ATP (2 mM, final concentration). At the indicated times, 100 μl aliquots were filtered through Millipore filters (see Methods); filters were washed twice with 3 ml of ice-cold solution containing 250 mM KCl/50 mM K-Mops (pH 7.3), dried and dissolved in Instagel. Controls were run in the absence of ATP and unspecific calcium binding was subtracted from each experimental point. Results obtained with three different preparations are expressed as mean ± S.E.

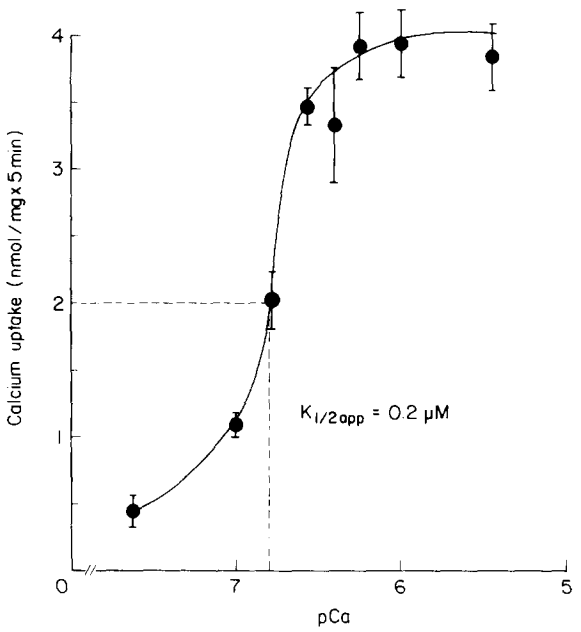

Fig. 2. ATP-dependent calcium uptake as a function of extravesicular free calcium concentration. Aliquots of membrane vesicles (0.4 mg protein/ml) were preincubated 5 min at 25°C in a medium containing (mM): 0.5 $MgCl_2$, 0.1 ouabain, 5 sodium azide, 0.5 EGTA-Tris, 100 KCl, 200 K-Mops (pH 7.3) and the quantities of $CaCl_2$ calculated to obtain the represented p*Ca* (see Methods) (^{45}Ca activity was $5 \cdot 10^5$ cpm/tube). Ca^{2+} uptake was initiated by adding ATP (2 mM, final concentration) and lasted 5 min. The reaction was stopped by filtration of 100 μl aliquots through Millipore filters. Calcium binding obtained in the absence of ATP was subtracted from each experimental point. Results from three different preparations expressed as mean ± S.E.

state was reached caused a rapid and complete release of the accumulated Ca^{2+}. This indicates that Ca^{2+} was transported against its concentration gradient and into the intravesicular space. This ATP-dependent Ca^{2+} uptake was not affected by inclusion of oligomycin or sodium azide in the incubation medium.

The relationship between free Ca^{2+} concentration and the operation of the ATP-dependent Ca^{2+} pump is shown in Fig. 2. The pumping activity increased with increasing free Ca^{2+} concentrations ranging from 0.01 to 1 μM. Free Ca^{2+} concentrations higher than 2 μM did not produce any further increment in the Ca^{2+} uptake, which reached a maximal rate of about 1 nmol/mg protein per min. A linear plot of Ca^{2+} data measured with 5 min incubations, during which the uptake was linear with time, revealed an apparent K_m of 0.2 μM.

The activation of Ca^{2+} uptake by ATP was explored by varying the concentration of the nucleotide in the presence of 1 mM $MgCl_2$ and 5 μM free Ca^{2+}. To stabilize the levels of ATP during the 5 min incubation period, an ATP regenerating system composed of phospho*enol*-pyruvate and pyruvate kinase was utilized. Fig. 3 shows that no net Ca^{2+} accumulation occurred in the absence of ATP. In the range from 10 to 100 μM ATP, net Ca uptake increased along a rectangular hyperbola and reached a maximum at approx. about 100 μM ATP. Half-maximal Ca uptake was observed at an ATP concentration of about 21 μM. This indicates that the Ca transport system is activated by ATP with high affinity, a result which agrees with the value obtained for the ATP-dependent Ca^{2+} transport in squid axons and for the (Ca^{2+} + Mg^{2+})-ATPase from the same preparation [2,11].

We also could demonstrate the presence of ATP-dependent Ca uptake using a calcium-selective microelectrode. Fig. 4 shows a typical experi-

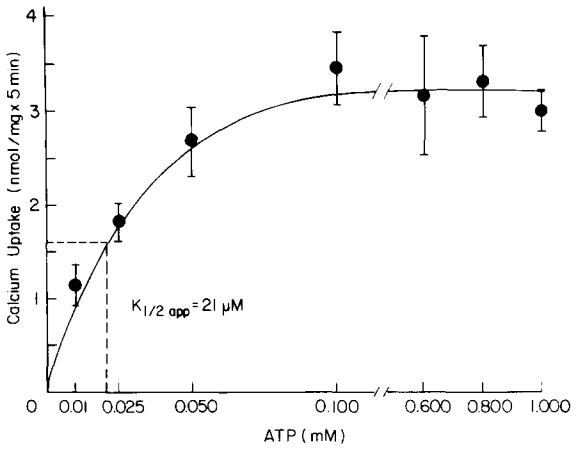

Fig. 3. Effect of different ATP concentrations on the active calcium uptake by nerve membrane vesicles. Aliquots of membrane vesicles (0.3 mg protein/ml) were preincubated 5 min at 25°C in a medium comprising (in mM) 1 $MgCl_2$, 0.1 ouabain, 5 Na-azide, 0.5 EGTA-Tris, 100 KCl, 200 K-Mops, 1 phospho*enol*pyruvate 1 U/ml pyruvate kinase (pH 7.3) and quantities of $^{45}CaCl$ adequate to obtain $[Ca^{2+}] = 5$ μM for each concentration of ATP. The reaction was initiated by addition of ATP and arrested after 5 min by Millipore filtration of 100 μl aliquots. Controls were run in the absence of ATP and unspecific Ca binding was subtracted from each experimental point. Results obtained with three different preparations expressed as mean ± S.E.

ment, which continuously monitored ATP-dependent Ca loss from the medium by using a calcium microelectrode which was calibrated in a medium of high ionic strength (0.3). The contaminating Ca^{2+} concentration (10 μM) was used as the initial concentration of the incubating medium. Neither ATP alone nor vesicles plus the ATP regenerating system caused a significant change in the ionized Ca of the medium. However, when ATP was added in the presence of all other components, a continuous decrease in the ionized calcium concentration of the medium was observed, levelling off in this particular experiment after about 5 min. As was observed in the isotope uptake experiments, addition of the Ca ionophore A23187 induced complete release of the accumulated Ca.

Orthovanadate inhibits both the ATP-dependent Ca uptake of dialyzed squid axons [17] and $(Ca^{2+} + Mg^{2+})$-ATPase activity of isolated nerve membranes [11]. Ca uptake present in nerve membrane vesicles was also inhibited by orthovanadate in a dose-dependent manner. Preincubation of the vesicles in orthovanadate (at 25°C) prior to addition of ATP lowered the concentration required

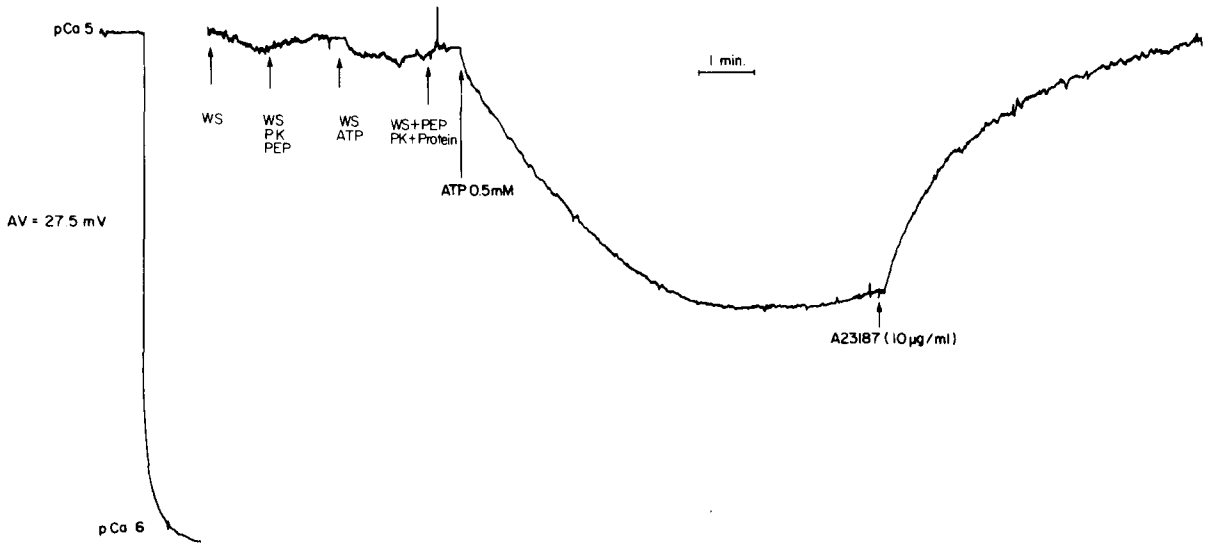

Fig. 4. ATP-dependent calcium uptake measured by means of Ca-selective microelectrodes. Ca^{2+} uptake was carried out in 0.1 ml of a medium comprising (in mM) 1.5 $MgCl_2$, 0.1 ouabain, 100 KCl, 200 K-Mops (wash solution, WS in figure), plus 0.5 phospho*enol*pyruvate, 1 U/ml pyruate kinase and 1 mg/ml membrane protein, and was initiated by addition of 0.5 mM ATP-Tris (final concentration). Initial extravesicular free calcium concentration was 10 μM.

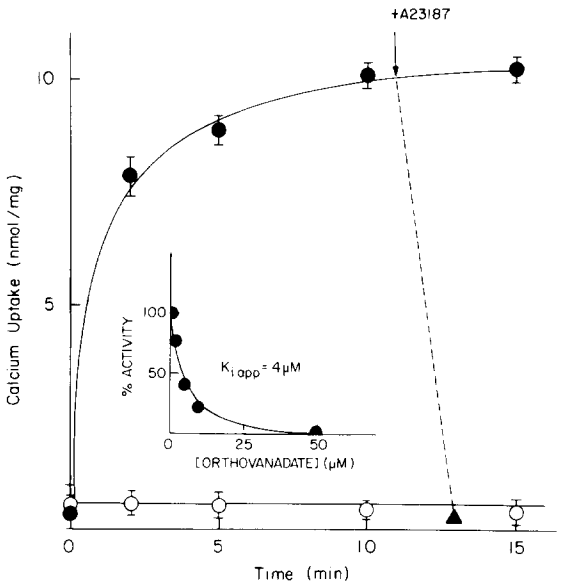

Fig. 5. Effect of orthovanadate on the ATP-dependent calcium uptake by nerve membrane vesicles. Aliquots of membrane vesicles (0.25 mg protein/ml) were preincubated 5 min at 25°C in a medium comprising (in mM) 0.5 $MgCl_2$, 0.1 ouabain, 5 sodium azide, 0.5 EGTA-Tris, 150 KCl, 150 NMG-Mops and 0.605 $^{45}CaCl_2$, in the presence (○) or not (●) of 100 μM orthovanadate. Calcium uptake was initiated by addition of 2 mM ATP. At indicated times, 100 μl of vesicles were filtered through Millipore filters. Inset: in order to calculate the apparent K_i for orthovanadate, vesicles were allowed to take calcium for 10 min in the presence of different concentrations of this compound. The uptake capacity measured in the presence of each concentration was expressed as a percentage of the total uptake capacity obtained in the absence of orthovanadate (100% activity).

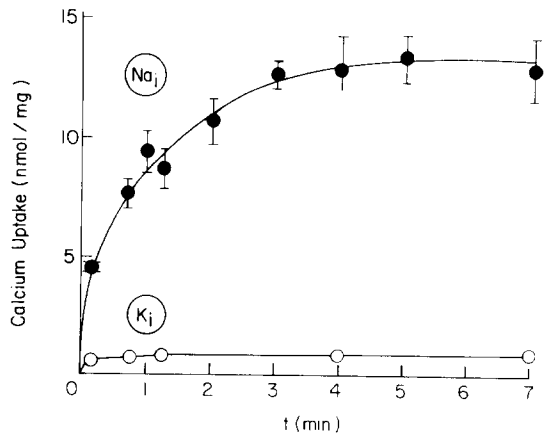

Fig. 6. Time-course of calcium uptake by Na^+- or K^+-preloaded vesicles. Vesicles (7 mg/ml) were preincubated 30 min at 37°C in media containing (mM): (●) 100 NaCl, 200 Na-Mops (pH 7.3) at 37°C), and (○) 100 KCl, 200 K-Mops (pH 7.3 at 37°C). Calcium uptake was initiated by diluting 50 μl of preloaded vesicles in 1 ml of (mM): 100 *N*-methyl D-glucamine chloride, 0.1 ouabain, 0.1 $^{45}CaCl_2$ (^{45}Ca sp. act. $2.4 \cdot 10^4$ cpm/nmol, 200 NMG-Mops (pH 7.3). At indicated times, after incubation at 25°C, 100 μl aliquots were filtered through Millipore filters. Results from four different preparations (Na-loaded vesicles) and two different preparations (K^+-loaded vesicles) were expressed as mean ± S.E.

for half-maximum inhibition from 35 μM to 4 μM, as has been described for other preparations [9,18]. Fig. 5 shows a Ca-uptake experiment realized in the absence or in the presence of 100 μM orthovanadate. A dose-response curve is presented in the inset showing an apparent K_i for orthovanadate of 4 μM.

Na^+-dependent Ca^{2+} uptake

Squid optic nerve vesicles were next assayed for Na^+-dependent Ca^{2+} uptake. In these experiments, vesicles (5–7 mg/ml) were passively equilibrated for 30 min at 37°C in either Na-Mops or K-Mops (300 mM) and aliquots were then diluted 20-fold into a medium containing 100 μM Ca^{2+}. Sodium or potassium was replaced in the dilution medium by the impermeant cation, NMG. Controls were prepared by diluting the preloaded vesicles in a medium containing either 300 mM Na^+ or K^+ in order to collapse the respective gradient and the results obtained in these conditions were then subtracted from those obtained in the presence of the monovalent cation gradient. Fig. 6 shows the time-course of Ca uptake into vesicles preloaded with either sodium or potassium. In the Na^+-loaded vesicles, calcium uptake occurred rapidly during the first minute, and reached a saturating value of 12–14 nmol/mg protein within 4 min. In contrast, vesicles preloaded with K^+ were unable to accumulate significant amounts of Ca. In the following experiments, the initial rates of Ca uptake were estimated during the first 10 s from the initial slope of the uptake curve shown in Fig. 6. The effect of different intravesicular Na^+ concentrations on the net Ca uptake was examined in vesicles loaded with sodium for 30 min at 37°C and then diluted in a Na^+-free medium containing ^{45}Ca (100 μM), as

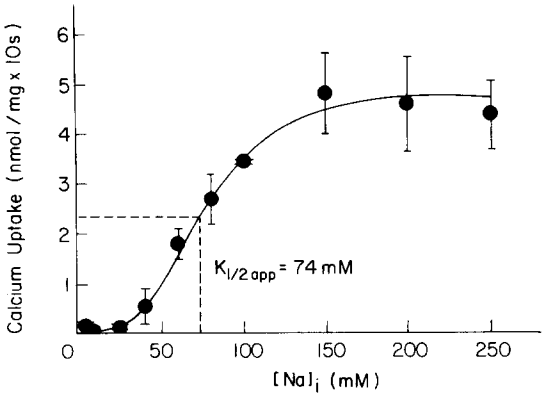

Fig. 7. Effect of different concentrations of intravesicular sodium on the calcium uptake by nerve membrane vesicles. Vesicles (5 mg protein/ml) were preincubated 30 min at 37°C in media comprising (mM) 5–250 NaCl, 245–0 NMG-Cl, 50 NMG-Mops (pH 7.3). Reaction was initiated by diluting 30-fold 5 μl of preloaded vesicles in (mM) 250 NMG-Cl, 0.1 ouabain, 0.1 $^{45}CaCl_2$ (^{45}Ca sp. act. $1.3 \cdot 10^4$ cpm/nmol), 50 NMG-Mops (7.3). After a 10 s incubation at 25°C, 100-μl aliquots were filtered through Millipore filters. Results obtained with four different preparations are expressed as mean ± S.E.

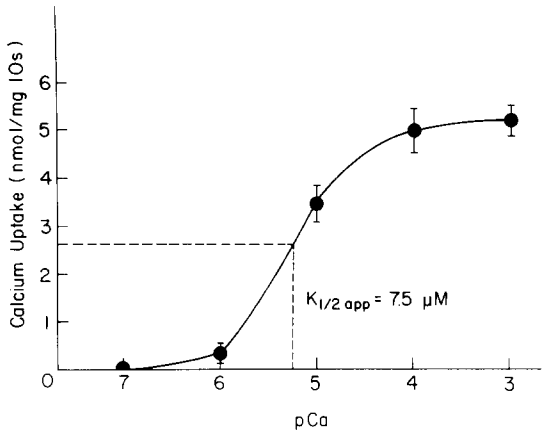

Fig. 8. Calcium uptake as a function of extravesicular Ca^{2+} concentration. Vesicles (5 mg protein/ml) were preloaded for 30 min at 37°C in a medium comprising (mM) 200 NaCl, 50 NMG-Cl, 50 NMG-Mops (pH 7.3) and calcium uptake was initiated by diluting 30-fold 5 μl of preloaded vesicles in (mM) 250 NMG-Cl, 0.1 ouabain, 50 NMG-Mops (pH 7.3), 0.5 EGTA, HEEDTA or nitrilotriacetic acid (see Methods) and adequate quantities of $CaCl_2$ calculated to obtain the represented p*Ca* (sp. act. ^{45}Ca, $5 \cdot 10^5$ cpm/tube). After a 10 s incubation at 25°C, 100-μl aliquots of the reaction mixture were filtered through Millipore filters. Results obtained with three different preparations are expressed as mean ± S.E.

shown in Fig. 7. In order to maintain a constant ionic strength and osmolarity, sodium was replaced by NMG when required. Vesicles preloaded with 5 mM NaCl showed negligible net Ca accumulation, but as the Na concentration was increased, Ca uptake rose until it reached a V_{max} of 27 nmol/min per mg protein for a Na concentration of 150 mM. The calculated apparent affinity constant for Na^+ of the Na^+-dependent Ca uptake was 74 mM. Control values obtained in the absence of internal Na (1 nmol/mg) were subtracted from each experiment point. The sigmoidal nature of this curve suggests that more than one sodium is required for the translocation of one calcium ion.

A similar kinetic characterization of the Ca^{2+} transport, but as a function of extravesicular Ca^{2+}, is shown in Fig. 8. In this case vesicles were preloaded with a saturating Na^+ concentration (200 mM) and uptake initial rates were determined. The data indicate that the apparent K_m for Ca^{2+} is 7.5 μM and the V_{max} of uptake 27 nmol/min per mg protein. These kinetic constants are comparable to those reported for the Na^+-Ca^{2+} exchange in brain and purified heart membrane vesicle preparations [20–22].

Discussion

There is general agreement that in most excitable cells two co-functional transport systems are responsible for active Ca translocation: an ATP-dependent Ca-pump and the Na^+-Ca^{2+} exchanger. The functional significance of these two mechanisms has been controversial with respect to their role in the regulation of the physiological internal free calcium concentration. Although the affinities and capacities for Ca transport of these two systems vary in different tissues, most of the present knowledge indicates that the Ca pump, due to its high affinity but lesser capacity, must be responsible for the resting internal ionized calcium.

In this study, an ATP-dependent Ca^{2+} transport system and a Na^+-Ca^{2+} exchange have been identified and characterized in plasma membrane vesicles derived from squid optic nerves.

Several lines of evidence indicate that these activities are located in the plasma membrane. ATP-dependent Ca^{2+} uptake is not sensitive to inhibition by oligomycin or sodium azide, besides,

the preparation is enriched in plasma membrane enzyme markers and virtually lacks mitochondrial markers activity [10,11]. Taken together, these results indicate that Ca^{2+} uptake is not due to contamination of the preparation with vesicles derived from mitochondrial membrane. On the other hand, the lack of effect of oxalate on Ca^{2+} accumulation (unpublished results) strongly argues against the possibility that the origin of this activity might be a contamination by endoplasmic reticulum membranes.

Ca^{2+} is accumulated inside the vesicles, rather than just bound to the membrane, because accumulation takes place against the Ca electrochemical gradient and exposure to the Ca ionophore A23187 causes accumulation to cease and release of accumulated calcium.

Using an average vesicular volume of 1.7 μl/mg protein it may be inferred that at steady state a maximal intravesicular Ca^{2+} content of about 8.3 nmol/mg protein is attained, which represents a concentration of about 4.9 mM, about 10^3-times the Ca^{2+} concentration in the extravesicular solution.

The kinetic properties of the ATP-dependent Ca^{2+}-accumulation are similar to those described for the Ca^{2+}-pump present in a variety of excitable and nonexcitable cells [2,5,8,9]. The affinity for calcium of the active Ca^{2+} uptake studied in optic nerve membrane vesicles (0.2 μM) closely parallels that of the (Ca^{2+} + Mg^{2+})-ATPase of the same preparation (0.12 μM) and of the ATP-dependent uncoupled Ca^{2+} efflux operating in dialyzed squid axons (0.18 μM). This strongly suggests that ATP-dependent Ca^{2+} accumulation in membrane vesicles is a reflection of the uncoupled Ca pump reported in axons and is due to the activity of the (Ca^{2+} + Mg^{2+})-ATPase. The (Ca^{2+} + Mg^{2+})-ATPase present in squid optic nerve membranes hydrolyze about 12 nmol ATP/min per mg protein at 25°C under high ionic strength conditions [10]. On the other hand, the average V_{max} of the ATP-driven Ca^{2+} uptake measured under similar conditions is only 1 nmol Ca^{2+}/min per mg protein. However, this V_{max} is obviously underestimated, since only the inside-out vesicles would be expected to function in the case of ATP-dependent Ca^{2+} transport, and this fraction represents only a 10% of the total population. Hence, Ca^{2+} transport rates via the ATPase pathway would be 10-fold higher. It is evident that a correlation exists between ATPase enzymatic activity and Ca^{2+} transport and a high degree of coupling of ATP hydrolysis to Ca^{2+} transport may be detected.

ATP-dependent Ca^{2+} transport in squid optic nerve vesicles is inhibited by orthovanadate with high affinity (app. $K_i = 4$ μM). It has been reported in a variety of cells (including squid axons) that this compound inhibits Ca^{2+} active transport with comparable affinity [8,9,19].

The experiments described in this paper also present evidence for Na^+-Ca^{2+} exchange in optic nerve membrane vesicles. This system is, in many respects similar to the exchange characterized in dialyzed squid axons [23] and in vesicles from other cell types [20–22,24–27]. In optic nerve vesicles, Na^+-Ca^{2+} exchange displays an apparent K_m for Ca^{2+} more than one order of magnitude higher than that corresponding to ATP-dependent Ca^{2+} pump, as observed in squid axons. The average V_{max} of the exchange is 27 nmol/min per mg of protein in our preparation, which is approx. 3-fold that of the active Ca^{2+} transport corrected for inside-out vesicles. In squid axons, DiPolo et al. reported that the V_{max} of the Na^+-Ca^{2+} exchange measured in the absence of ATP is approx. 5-fold the V_{max} of the uncoupled Ca^{2+} pump [23]. Thus it appears that Na^+-Ca^{2+} exchange is a low affinity but high capacity Ca transport system. The translocation of Ca^{2+} via the Na^+-Ca^{2+} exchanger requires a maintained Na^+ gradient driving force. However, these vesicles show a relatively high permeability for Na^+ (not shown) and due to Na^+ gradient dissipation during the time-course of the reaction the initial rate of the Ca^{2+} uptake through the exchanger may be underestimated.

In conclusion, our results indicate that optic nerve plasma membrane from squid axons contains the two functional Ca^{2+} transport mechanisms that have been demonstrated in intact axons and implicated in the control of the intracellular free Ca^{2+} levels. This paves the way for detailed biochemical analysis of these two transport molecules in this system.

Acknowledgments

We thank Mrs. Rebeca Godoy for her secretarial help, Mrs. Dhuwya Palma for the figures and the squid supply staff at IVIC and Mochima. This work was supported by CONICIT (S1-1144 and RH 15-123), Venezuela. L.O. was the recipient of a Fundación Vollmer fellowship.

References

1 DiPolo, R. (1978) Nature (Lond) 274, 390–392
2 DiPolo, R. and Beaugé, L.A. (1979) Nature (Lond.) 278, 271–273
3 Caroni, P. and Carafoli, E. (1980) Nature (Lond.) 283, 765–767
4 Reuter, H. and Seitz, N. (1968) J. Physiol. (Lond.) 195, 451–470
5 Penniston, J.T. (1982) Ann. N.Y. Acad. Sci. 402, 296–303
6 Kaczorowski, G.J., Vandlen, R.L., Katz, G.M. and Reuben, J.R. (1983) J. Membrane Biol. 71, 109–118
7 Blaustein, M.P. (1977) Biophys. J. 20, 79–111
8 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263–3270
9 Barros, F. and Kaczorowski, G.J. (1984) J. Biol. Chem. 259, 9404–9410
10 Beaugé, L., DiPolo, R., Osses, L., Barnola, F. and Campos, M. (1981) Biochim. Biophys. Acta 644, 147–152
11 Condrescu, M., Osses, L. and DiPolo, R. (1984) Biochim. Biophys. Acta 769, 261–269
12 Osses, L., Condrescu, M. and DiPolo, R. (1984) Biophys. J. 45, 79a
13 Padan, E., Silberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533–541
14 Fiske, C.H. and SubbaRow, Y.T. (1925) J. Biol. Chem. 66, 375–400
15 Caroni, P. and Carafoli, E. (1983) Eur. J. Biochem. 132, 451–460
16 Brinley, F.J., Scarpa, A. and Tiffert, T. (1977) J. Physiol. 226, 545–564
17 DiPolo, R., Rojas, H. Vergara, J., López, R. and Caputo, C. (1983) Biochim. Biophys. Acta 728, 311–318
18 DiPolo, R. and Beaugé, L. (1981) Biochim. Biophys. Acta 645, 229–236
19 Wang, T., Tsai, L., Solaro, R.J., Grassi de Gende, A.D. and Schwartz, A. (1979) Biochem. Biophys. Res. Commun. 91, 356–361
20 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 590–594
21 Bers, D.M., Philipson, K.D. and Nishmoto, A.Y. (1980) Biochim. Biophys. Acta 601, 358–371
22 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232–6235
23 DiPolo, R. and Beaugé, L. (1983) Annu. Rev. Physiol. 45, 313–324
24 Gill, D.L., Grollman, E.F. and Kohn, L.D. (19181) J. Biol. Chem. 256, 184–192
25 Philipson, K.D. and Nishimoto, A.Y. (1982) J. Biol. Chem. 257, 5117
26 Schellenberg, G.D. and Swanson, P.D. (1981) Biochim. Biophys. Acta 648, 13–27
27 Kaczorowski, G.J., Costello, L., Dethmers, J. Trumble, M.J. and Vandlen, R.L. (1984) J. Biol. Chem. 259, 9395–9403