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PARTIAL PURIFICATION AND CHARACTERIZATION OF THE $(Ca^{2+} + Mg^{2+})$-ATPase FROM SQUID OPTIC NERVE PLASMA MEMBRANE

MADALINA CONDRESCU, LUIS OSSES and REINALDO DiPOLO

Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, IVIC, Apartado 1827, Caracas 1010 A (Venezuela)

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A membrane fraction enriched in axolemma was obtained from optic nerves of the squid (*Sepiotheutis sepioidea*) by differential centrifugation and density gradient fractionation. The preparation showed an oligomycin- and NaN_3-insensitive $(Ca^{2+} + Mg^{2+})$-ATPase activity. The dependence of the ATPase activity on calcium concentration revealed the presence of two saturable components. One had a high affinity for calcium ($K^1_{1/2} = 0.12$ μM) and the second had a comparatively low affinity ($K^2_{1/2} = 49.5$ μM). Only the high-affinity component was specifically inhibited by vanadate ($K_i = 35$ μM). Calmodulin (12.5 μg/ml) stimulated the $(Ca^{2+} + Mg^{2+})$-ATPase by approx. 50%, and this stimulation was abolished by trifluoperazine (10 μM). Further treatment of the membrane fraction with 1% Nonidet P-40 resulted in a partial purification of the ATPase about 15-fold compared to the initial homogenate. This $(Ca^{2+} + Mg^{2+})$-ATPase from squid optic nerve displays some properties similar to those of the uncoupled Ca^{2+}-pump described in internally dialyzed squid axons, suggesting that it could be its enzymatic basis.

Introduction

Experiments with intact and dialyzed squid axons have demonstrated that besides the classical Na^2-Ca^{2+}-exchange mechanism nerve cells possess an ATP-driven Ca^{2+}-pump that is the main one responsible for maintaining a large electrochemical calcium gradient across the axonal membrane under resting conditions [1,2]. Therefore, an adequate enzyme preparation mainly proceeding from axonal membrane would be very useful to identify and characterize the biochemical counterpart of this Ca^{2+}-pump.

In several previous studies a $(Ca^{2+} + Mg^{2+})$-ATPase activity has been described in mammalian brain preparations and its relevance to the active transport of Ca^{2+} has been pointed out [3–7]. However, the subcellular localization of this enzyme was not clearly established and it is not known if it participates in the extrusion of Ca^{2+} from the cell across the plasma membrane or in the sequestration of this cation within intracellular reservoirs. In addition, this ATPase had a marked instability, showing half-lives of hours in most of the cases [3–5]. For a number of reasons, the optic nerves of the squid represent a particularly suitable material, which yields numerous advantages such as: no myelin contamination; low traces of subcellular organelles; and a favorable ratio of axonal over glial cell membranes [8]. Recently, a $(Ca^{2+} + Mg^{2+})$-ATPase activity has been reported in membrane fragments from optic nerves of the squid *Sepiotheutis sepioidea* [9].

Abbreviations: EGTA, ethyleneglycol bis(aminoethyl ether)-*NN'*-tetraacetic acid; PMSF, phenylmethylsulfonylfluoride; Nonidet P-40, octylphenoxypolyethoxyethanol; DTNB, 5.5'-dithiobis(2-nitrobenzoic acid); W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; Amidol, 2,4-diaminophenol dihydrochloride; Mops, 4-morpholinepropanesulphonic acid.

In the present study we have attempted to partially purify and further characterize the (Ca^{2+} + Mg^{2+})-ATPase from axonal membranes of squid optic nerves. This preparation shows a high specific activity and high yield and is stable for months when stored at −80°C, characteristics which makes it a valuable candidate for future biochemical studies of the ATP-driven Ca^{2+}-pump present in the axonal membrane.

Methods

Tissue source

Squids (*S. sepioidea*) were caught at Mochima bay (Venezuela) and after decapitation the heads were washed, stored in ice-cold, calcium-free artificial seawater, and transported to IVIC. The optic nerves were dissected out and stored overnight in 0.75 M sucrose/10 mM Tris (pH 7.4) at −20°C. Usually 30–50 squids were processed, yielding an average of 0.5 g of wet nerve tissue per squid.

In two cases, live squids were brought to the laboratory and the optic nerves were dissected out immediately. No difference in the (Ca^{2+} + Mg^{2+})-ATPase, (Na^+ + K^+)-ATPase and Mg^{2+}-ATPase activity and stability was observed when these preparations were compared to those made up from material processed as described above.

Preparation of plasma membrane

The steps involved in the isolation of plasma membrane represent a modification of the method described by Balerna et al. [10]. The nerves were slowly thawed to 4°C and washed in 0.32 M sucrose/1 mM EDTA/10 mM Tris (pH 7.4). The material was homogenized in the above solution containing in addition 1 mM PMSF (8 ml solution/g of wet tissue), by five strokes in a Teflon-glass homogenizer (5000 rpm) followed by five strokes (by hand) in a Dounce-glass homogenizer.

The suspension was centrifuged 8 min at 2000 × *g* in a Sorvall refrigerated centrifuge, the supernatant was separated and the pellet was washed twice. The combined supernatants were centrifuged 25 min at 12000 × *g* in the same centrifuge, washing twice the pellet and separating the supernatants. It is important to stress that the two-fold wash of the pellets in this and the previous step is crucial to obtain maximum yield.

A microsomal pellet was obtained by centrifugation of the combined supernatants proceeding from the 12000 × *g* step, for 45 min at 100000 × *g*, in a Beckman L8-55 ultracentrifuge using a 60 Ti rotor. In order to obtain further purification, this pellet was resuspended in 0.32 M sucrose/1 mM EDTA/10 mM Tris (pH 7.4), layered over 1.12 M sucrose/1 mM EDTA/10 mM Tris (pH 7.4) and centrifuged at 65000 × *g* for 90 min.

The interphase was collected, diluted 5-times with 10 mM Tris (pH 7.4) and sedimented at 100000 × *g*. This final pellet enriched in plasma membrane, was resuspended in 0.3 M sucrose/15 mM Tris (pH 7.4) (to about 5 mg protein/ml) and 0.2 ml aliquots were frozen in solid CO_2/acetone. The membrane was stored at −80°C and no loss in (Ca^{2+} + Mg^{2+})-ATPase, (Na^+ + K^+)-ATPase or Mg^{2+}-ATPase activity was observed over a period of at least 1 month.

The usual yield of the method was 3 mg of membrane protein per g of wet nerve tissue.

Nonidet P-40 treatment

The membrane fraction (1 mg/ml) was suspended in 0.3 M sucrose/15 mM Tris (pH 7.4). Nonidet P-40 was added very slowly from a 10% solution under stirring until a final concentration of the detergent of 1% (w/v) was reached. Incubation lasted 20 min at room temperature. The mixture was then diluted 15-fold with ice-cold sucrose/Tris solution and centrifuged for 30 min at 150000 × *g*. The insoluble pellet was resuspended in a minimum volume of 0.3 M sucrose/15 mM Tris (pH 7.4).

(Ca^{2+} + Mg^{2+})-ATPase assay

Assays were carried out in 1 ml comprising 2 mM Tris-ATP/0.5 mM $MgCl_2$/100 mM KCl/0.5 mM EGTA/50 mM histidine/0.1 mM ouabain/10 μg oligomycin (or 5 mM NaN_3) (pH 7.3). The (Ca^{2+} + Mg^{2+})-ATPase was taken as the difference between the activity observed in the presence of 600 μM $CaCl_2$ (11 μM free calcium) and that found with no added $CaCl_2$. The free calcium concentrations were calculated from the multiple equilibria involving ATP, EGTA, Ca and Mg. The dissociation constants used in the calculation were directly measured using a calcium electron connected to a microprocessor ionalyzer (Orion, model

901, Cambridge, MA) or estimated for our conditions of ionic strength, pH and temperature. The values were: Ca · ATP, 0.167 mM (measured), Ca · EGTA, 0.16 μM (measured), Mg · ATP, 28 μM (calculated for pH 7.3 from $\log K_a = 4.65$ [31]) and Mg · EGTA, 10 mM [32]. Normally, experiments were carried out in the presence of 0.5 mM $MgCl_2$ in order to minimize the contribution of the Mg^{2+}-ATPase to ATP hydrolysis, although 0.1 mM $MgCl_2$ was used in a number of experiments as pointed out in figure or table legends. No difference was observed in the $(Ca^{2+} + Mg^{2+})$-ATPase activity depending on $MgCl_2$ concentration in a range from 0.1–3 mM. Assay temperature of 37°C and the ionic strength have been purposely chosen to increase the hydrolysis rate to 4-times the rate measured at 25°C and at artificial seawater ionic strength. Nevertheless, these conditions did not affect the affinity of the enzyme for calcium. The reaction was initiated by adding 25–50 μg of membrane protein and incubation lasted for 15–30 min. Released inorganic phosphate was measured by a modification of the method of Fiske and SubbaRow [11], which uses Na_2SO_3 with amidol as reducing solution, or when [γ-^{32}P]-ATP was employed, with isobutanol extraction of the ^{32}P radioactivity. When the effect of calmodulin was tested, the incubation was carried out in 0.2 ml of the following medium, comprising 1.014 mM Tris-ATP/0.1 μCi/ml [γ-^{32}P]-ATP/3.338 mM $MgCl_2$/100 mM KCl/0.1 mM ouabain/10 μg/ml oligomycin/50 mM histidine (pH 7.3), in the absence of added calcium or in the presence of 6 μM $CaCl_2$. In these conditions, the free calcium concentration was 5 μM and that of Mg · ATP was 1 mM [33]. The reaction was started by adding 10 μg of membrane protein and incubation lasted for 10 min at 37°C. 12.5 μg/ml of bovine brain calmodulin was added to some of the tubes, while 12.5 μg/ml calmodulin plus 10 μM trifluoperazine were present in others.

Assay of marker enzymes

$(Na^+ + K^+)$-ATPase was assayed in 1 ml of medium comprising 3 mM Tris-ATP/3 mM $MgCl_2$/130 mM NaCl/20 mM KCl/1 mM EGTA/50 mM histidine (pH 7.3). Control tubes contained 0.1 mM ouabain. The reaction was initiated by adding 10 μg of membrane protein and incubation at 37°C lasted for 10 min. The liberated phosphate was quantified by the modified Fiske and SubbaRow method [11], 5′-Nucleotidase was assayed by the method of Avruch and Wallach [12] with 5′-AMP as substrate. Acetylcholine esterase was measured spectrophotometrically according to Ellman et al. [13] using 1 μM physostigmine in controls. Assays were performed in fresh fractions, since it was observed that after storage for 1 week at −80°C this particular enzyme activity was inhibited up to 60%, in contrast to the $(Ca^{2+} + Mg^{2+})$-ATPase, $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase, which were stable in these conditions. Succinate dehydrogenase was measured spectrophotometrically by a modification of the procedure of Hiatt [14] and glucose-6-phosphatase was determined according to Hübscher and West [15]. Protein was measured by the method of Lowry et al. [16], using bovine serum albumin as the standard.

Calmodulin radioimmunoassay

Samples for calmodulin radioimmunoassay were prepared according to Wallace and Cheung [28]; 0.6 g of squid optic nerves were homogenized in 3 ml of 50 mM Tris (pH 7.4), 3 mM $MgCl_2$/1 mM β-mercaptoethanol/1 mM EGTA. Five strokes in a Teflon-glass homogenizer (5000 rpm) were followed by five strokes by hand in a Dounce-glass homogenizer. The homogenate was centrifuged at $27\,000 \times g$ for 20 min in a Sorvall refrigerated centrifuge and the supernatant was recentrifuged for 1 h at $100\,000 \times g$ (Beckman L8-55 ultracentrifuge). The pellet obtained in this step was resuspended in 0.5 ml of the above buffer and represents the microsomal fraction, while the supernatant was heated to 95°C for 4.5 min and the denatured proteins were removed by centrifugation for 1 h at $100\,000 \times g$. The resulting supernatant (2.5 ml) was diluted 10-fold with buffer and an appropriate volume was used to determine the calmodulin concentration in the soluble fraction. In order to measure the membrane-bound calmodulin, an aliquot of the plasma membrane fraction (1 mg approx) was resuspended in 0.2 ml of the above buffer and 50 μl were used for each assay. ^{125}I-labeled calmodulin was used as the tracer and a specific sheep anti-calmodulin as the binder (New England Nuclear radioimmunoassay

kit). The assay buffer was 125 mM borate (pH 8.4), 75 mM NaCl/1 mM EGTA/0.2% bovine serum albumin/0.1% NaN_3. Standards of bovine brain calmodulin from 0.31 to 20 ng and adequate controls were run in parallel with samples. The tubes were incubated overnight with anticalmodulin and 30 min with the second antibody and then were centrifuged at $5000 \times g$ for 15 min in an Eppendorf microcentrifuge.

All operations were carried out at 4°C. The supernatants were discarded and ^{125}I was counted in the pellets. The amount of calmodulin in the samples was determined by interpolation from the standard curve.

Ouabain, EDTA, EGTA, Nonidet P-40, bovine serum albumin, Glucose 6-phosphate, phenazinemethosulfate, 2,6-dichlorophenolindophenol, disodium succinate, DTNB and acetylthiocholine iodide were purchased from Sigma, St. Louis, MO. Oligomycin was from Calbiochem-Behring, La Jolla, CA, and physostigmine salicylate from E. Merck, F.R.G. Sodium orthovanadate was obtained from Fisher, Fair Lawn, NJ, [γ-^{32}P]ATP and the calmodulin ^{125}I-RIA kit were from New England Nuclear, Boston, MA. Bovine brain calmodulin was obtained from Fluka, Buchs, Switzerland and triofluoperazine was a gift from Laboratorios Klinos, Caracas, Venezuela. All other chemicals were reagent grade.

Results

Purity of the plasma membrane fraction

Electron microscopy and enzymatic techniques were used to evaluate the purity of the plasma membrane fraction: the electron microscopy of the negatively stained membrane revealed the presence of vesicles, as described already by Fischer et al. [8], Beaugé et al. [9], Balerna et al. [10] and Barnola et al. [17]. No indication of contamination by subcellular organelles was found by this method. Several marker enzymes have been tested along the fractionation process: $(Na^+ + K^+)$-ATPase, 5'-nucleotidase and acetylcholine esterase (plasma membrane), succinate dehydrogenase (mitochondria) and glucose-6-phosphatase (endoplasmic reticulum). Table I shows the distribution of these enzymes in various fractions isolated from total homogenates of optic nerves: it appears that 5'-nucleotidase, $(Na^+ + K^+)$-ATPase and acetylcholine esterase have a rather similar distribution, all of them showing the highest specific activity in the membrane fraction; 2.3–3.1-fold purification of these specific plasma membrane markers was achieved by the fractionation procedure, while 20–30% of their total activity was recovered in this fraction. Moreover, two lines of evidence suggest that the plasma membrane fraction contains mostly axonal membranes: (a) in *S. sepioidea* giant axons the acetylcholine esterase activity is located mainly at axolemma level [18]; and (b) in *Dosidicus gigas* optic nerves there is 10-times more excitable cell membrane than Schwann cell membrane [8].

The $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured along the fractionation and it is clear from Table I that it parallels the distribution of the plasma membrane specific markers, supporting the idea that it originates from plasma membrane and, in the light of the above arguments, from axonal membrane.

The specific activity of the mitochondrial marker enzyme succinic dehydrogenase in the membrane fraction was only 8% of that in the mitochondrial-enriched $12\,000 \times g$ pellet, indicating a low degree of mitochondrial contamination. The absence of inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity by oligomycin (10 μM) and NaN_3 (5 mM) indicates that this activity does not derive from contaminating mitochondria.

Since previous work suggested that squid axons endoplasmic reticulum captures calcium with a relatively high affinity [29,30], we evaluated the degree of contamination of the plasma membrane fraction by this organelle. The endoplasmic reticulum marker enzyme glucose-6-phosphatase was measured and we observed that this activity was spread among all fractions. In spite of this, one cannot argue that the $(Ca^{2+} + Mg^{2+})$-ATPase activity present in the plasma membrane fraction is an expression of the contaminant endoplasmic reticulum because while the glucose-6-phosphatase specific activity was about the same in the membrane and in the total homogenate (0.6 $\mu mol \cdot mg^{-1} \cdot h^{-1}$), the $(Ca^{2+} + Mg^{2+})$-ATPase specific activity was about 3-times higher in the membrane compared to the total homogenate. In addition, after treatment of the membrane with 1% Nonidet

TABLE I

DISTRIBUTION OF VARIOUS ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS FROM SQUID OPTIC NERVE

Values are given as averages of six different preparations. $(Ca^{2+} + Mg^{2+})$-ATPase was determined in the presence of 10 μM Ca^{2+} and 0.1 mM $MgCl_2$.

	Protein yield (%)	5'-Nucleotidase		$(Na^+ + K^+)$-ATPase		Acetylcholine esterase		$(Ca^{2+} + Mg^{2+})$-ATPase		Succinate dehydrogenase	
		nmol $\cdot P_i \cdot mg^{-1} \cdot h^{-1}$	Total units (%)	μmol $\cdot P_i \cdot mg^{-1} \cdot h^{-1}$	Total units (%)	nmol $\cdot mg^{-1} \cdot min^{-1}$	Total units (%)	μmol $\cdot P_i \cdot mg^{-1} \cdot h^{-1}$	Total units (%)	μmol $\cdot mg^{-1} \cdot h^{-1}$	Total units (%)
Total homogenate	100	4.29	100	40.1	100	21.8	100	1.15	100	0.26	100
2000 × *g* pellet	8	7.66	14	23.2	5	14.6	7	1.68	12	0.34	11
12000 × *g* pellet	19	8.29	37	50.5	24	35.3	33	0.73	12	0.62	45
Soluble fraction	64	0	0	6.3	10	4.9	14	0.10	6	0	0
Sucrose gradient pellet	2	2.7	1	56.6	3	23.6	6	1.18	2	0.32	3
Membrane fraction	10	10.4	24	125	31	50.7	19	3.40	30	0.05	2

TABLE II

DISTRIBUTION OF GLUCOSE-6-PHOSPHATASE ACTIVITY OVER THE PURIFICATION PROCESS
Mean values of results obtained with two different preparations.

Fraction	Protein yield (%)	Glucose-6-phosphatase specific activity ($\mu mol \cdot P_i \cdot mg^{-1} \cdot h^{-1}$)	Glucose-6-phosphatase total activity ($\mu mol \cdot P_i \cdot h^{-1}$)
Total homogenate	100	0.60	3.00
Membrane fraction	10	0.62	0.31
1% Nonidet P-40-treated membrane	1.8	0.31	0.03

P-40, the $(Ca^{2+} + Mg^{2+})$-ATPase specific activity was 15-fold increased with respect to the total homogenate, while the glucose-6-phosphatase specific activity was diminished to one half, without inactivation of this enzyme by the detergent per se (Table II). One more argument can be used to support the assumption that this calcium-stimulated ATPase activity could not originate from the endoplasmic reticulum: in experiments where the ATP-dependent Ca^{2+} uptake was studied in membrane vesicles, oxalate (5 mM) did not stimulate the uptake, as is the case with the endoplasmic reticulum vesicles [19] (Osses, L., unpublished data).

Nonidet P-40-treated membrane

In an attempt to enhance the $(Ca^{2+} + Mg^{2+})$-ATPase activity further by solubilizing or destroying other ATPase activities, treatment of the membrane with various detergents was tried. Nonidet P-40 produced the most promising results: incubation of the membrane with 1% Nonidet P-40 (w/v) in 0.3 M sucrose/15 mM Tris (pH 7.4) for 20 min at room temperature followed by dilution with sucrose/Tris and removal of the solubilized material by centrifugation resulted in a membranous preparation with 5-fold increment in the $(Ca^{2+} + Mg^{2+})$-ATPase specific activity (Table III). It should be noted that the detergent per se did not inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase, since the original total activity of the enzyme was recovered after detergent treatment. Mg^{2+}-ATPase specific activity was also increased around 5-fold by this treatment of the membrane, while $(Na^+ + K^+)$-ATPase was completely inhibited by 1% Nonidet P-40 (not shown). Like the native membrane, this preparation is stable for at least 1 month when stored frozen at $-80°C$. Attempts to solubilize the $(Ca^{2+} + Mg^{2+})$-ATPase still bound to the membrane have been unfruitful so far.

Activation of the $(Ca^{2+} + Mg^{2+})$-ATPase by Ca^{2+}

The activation by Ca^{2+} of the Ca^{2+}-stimulated ATPase was investigated in the native plasma membrane varying the free calcium concentration in the range from 10^{-8}–10^{-4} M. The curve turns

TABLE III

PARTIAL PURIFICATION OF $(Ca^{2+} + Mg^{2+})$-ATPase AND Mg^{2+}-ATPase BY TREATMENT WITH NONIDET P-40

Values given as mean ± S.E. of four different preparations. $(Ca^{2+} + Mg^{2+})$-ATPase was determined in the presence of 10 μM Ca^{2+} and 0.1 mM $MgCl_2$ and Mg^{2+}-ATPase was determined in the presence of 0.1 mM $MgCl_2$.

	Protein		$(Ca^{2+} + Mg^{2+})$-ATPase			Mg^{2+}-ATPase		
	μg	%	Specific activity ($\mu mol \cdot P_i \cdot mg^{-1} \cdot h^{-1}$)	Total activity ($\mu mol \cdot P_i \cdot h^{-1}$)	Purification	Specific activity ($\mu mol \cdot P_i \cdot mg^{-1} \cdot h^{-1}$)	Total activity ($\mu mol \cdot P_i \cdot h^{-1}$)	Purification
Control	500	100	3.1 ± 0.3	1.5 ± 0.2 ($n = 8$)	–	4.3 ± 0.8	2.1 ± 0.4 ($n = 8$)	–
1% Nonidet	78 ± 17	16	15.4 ± 3.3	1.2 ± 0.4 ($n = 8$)	5×	24.1 ± 7.2	1.6 ± 0.4 ($n = 8$)	5.6×

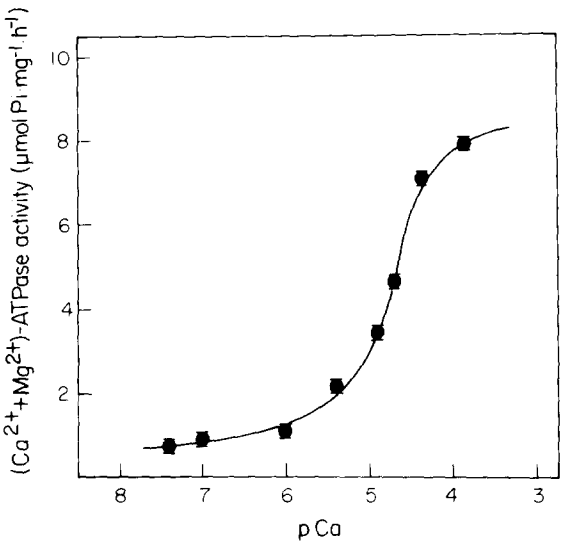

Fig. 1 Activation by Ca^{2+} of the $(Ca^{2+} + Mg^{2+})$-ATPase from membrane fragments of squid optic nerve. The medium contained 2 mM Tris-ATP/100 mM KCl/0.5 mM $MgCl_2$/0.5 mM EGTA/0.1 mM ouabain/ 5 mM NaN_3/50 mM histidine (pH 7.3). Enough $CaCl_2$ was added to obtain the desired free calcium concentrations, which were calculated from the multiple equilibria involving ATP, EGTA, Ca and Mg. The dissociation constants used in the calculation were measured or estimated for our conditions of ionic strength, pH and temperature. The values were: Ca-ATP, 0.167 mM (measured); Ca·EGTA, 0.16 μM (measured); Mg·ATP, 28 μM (calculated for pH 7.3 from $\log K_a = 4.65$ [31]); Mg·EGTA, 10 mM [32]. Each point is a mean ± S.E. of eight determinations.

out to be biphasic (Fig. 1), revealing the presence of two saturable components. Data from four different preparations were represented in an Eadie-Haldane plot showing a high-affinity component ($K^1_{1/2} = 0.12 \pm 0.06$ μM, $V_{max_1} = 1.34 \pm 0.17$ μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ ($n = 8$)) and a comparatively low-affinity component ($K^2_{1/2} = 49.5 \pm 3.7$ μM, $V_{max_2} = 8.8 \pm 0.4$ μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ ($n = 8$)).

In order to evaluate the effect of the ionic strength on the activation by Ca^{2+} of the enzyme, the experiment was repeated at seawater ionic strength (replacing 50 mM histidine by 200 mM potassium Mops in the assay medium). In these conditions, the affinity constants $K^1_{1/2} = 0.13 \pm 0.01$ μM ($n = 4$) and $K^2_{1/2} = 54.7 \pm 8.1$ μM ($n = 4$) were not different from those obtained at low ionic strength, while $V_{max_1} = 0.42 \pm 0.27$ μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ and $V_{max_2} = 3.23 \pm 0.75$ μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ were about one-half the corresponding maximal velocities at low ionic strength.

Calmodulin stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity

The calcium-dependent regulatory protein calmodulin is known to stimulate both the $(Ca^{2+} + Mg^{2+})$-ATPase and the ATP-dependent calcium uptake in a number of plasma membrane preparations [23,26,27]. In our system, purified bovine brain calmodulin stimulated by approximately 50% the $(Ca^{2+} + Mg^{2+})$-ATPase activity. In order to observe this stimulation a 'saturating' Mg^{2+} concentration in the millimolar range was needed. Furthermore, addition of the calmodulin inhibitor trifluoperazine (10 μM) fully antagonized the effect produced by calmodulin addition. This result agrees with a previous observation made in dialyzed squid axons where the ATP-driven Ca^{2+}-pump was inhibited by another calmodulin antagonist, W-7 (DiPolo and Beaugé, unpublished results). While these experiments suggest that endogenous calmodulin is present in squid axons and it could be implicated in the regulation of the Ca^{2+}-pump, we measured the calmodulin concentration in both whole optic nerves and in the membrane fraction. Samples were prepared according to Wallace and Cheung [28] and radioimmunoassay of calmodulin was carried out. We found that squid optic nerves contain 20.8 μg of calmodulin per g wet tissue; 98% of this was present in the soluble fraction, while the resting 2% was bound to microsomes. The membrane fraction contains an average of 8.9 ng calmodulin per mg of protein, representing only 5.3% of the microsome-bound calmodulin. This suggests that either a small fraction of the microsome-bound calmodulin is bound to the plasma membrane or the membrane has been depleted of calmodulin during the fractionation procedure.

Effect of vanadate

In dialyzed squid axons, orthovanadate has been reported to inhibit the uncoupled Ca^{2+} efflux, but not the Na^+-Ca^{2+} exchange. The K_i for this effect was 7 μM [24]. Previous work with red blood cells showed that K^+ and Mg^{2+} enhance the inhibitory effect of vanadate by increasing the affinity of the enzyme for this ion (K_i reaching 1.5 μM in the

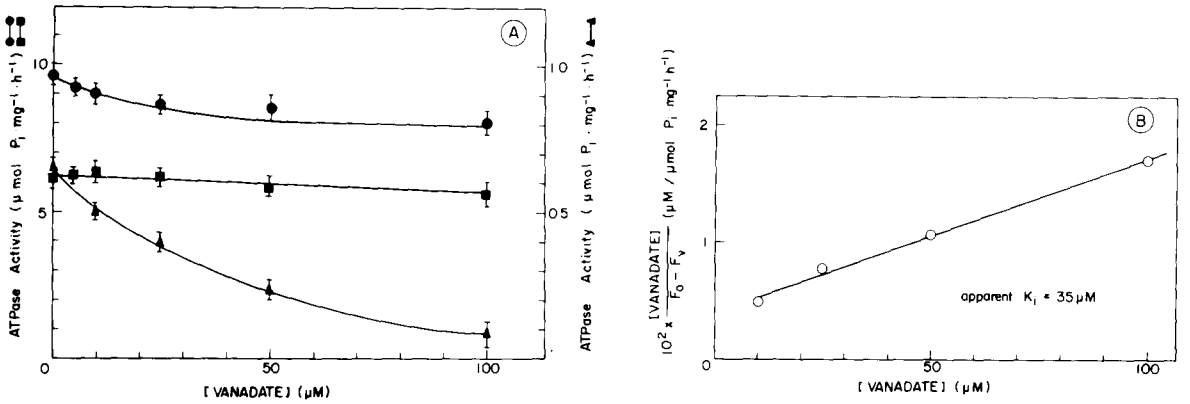

Fig. 2. Effect of vanadate on the $(Ca^{2+} + Mg^{2+})$-ATPase and Mg^{2+}-ATPase in membrane fragments of squid optic nerve. (A) The high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase activity (▲———▲) was taken as the difference between the activity measured in the presence of 1 μM free calcium and that determined in the absence of calcium (Mg^{2+}-ATPase, ●———●). The low-affinity component of the $(Ca^{2+} + Mg^{2+})$-ATPase (■———■) was taken as the difference between the activity measured in the presence of 100 μM free calcium and that obtained in the absence of calcium. Each point is a mean ± S.E. of three determinations. $MgCl_2$ concentration was 0.5 mM. (B) Scatchard fit of the data in (A) regarding the high-affinity component of the $(Ca^{2+} + Mg^{2+})$-ATPase. F_0 is the activity in control conditions and F_v in the presence of the corresponding concentration of vanadate.

presence of 60 mM K^+ and 10 mM Mg^{2+} [25]). The effect of vanadate on the $(Ca^{2+} + Mg^{2+})$-ATPase was tested in the presence of 1 μM Ca^{2+}, when the high-affinity component of the ATPase is apparent, or in the presence of 100 μM Ca^{2+} to observe its effect on the low-affinity component. Interestingly, only the high-affinity component of the $(Ca^{2+} + Mg^{2+})$-ATPase was inhibited by vanadate, while the low-affinity component remains virtually unaffected (Fig. 2A). In addition, Mg^{2+}-ATPase was only about 20% inhibited in the presence of 100 μM vanadate. A K_i of 35 μM was calculated for the vanadate inhibition of the high-affinity component of the $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 2B). The fact the we find an inhibition constant about one order of magnitude lower compared to previously reported data could be due to the low Mg^{2+} concentration present in our assay medium (0.5 mM $MgCl_2$), while high Mg^{2+} (4–10 mM $MgCl_2$) was used in these experiments.

Discussion

The $(Ca^{2+} + Mg^{2+})$-ATPase activity associated with this squid optic nerve plasma membrane preparation has the following several advantages for the study of the biochemical basis of the ATP-driven Ca^{2+}-pump from nerve cells. (1) Most of the work on $[Ca^{2+}]_i$ regulation in nerve cells has been realized in squid axons [1,2,24] and our preparation was enriched in axonal membranes. (2) The contamination by intracellular organelles was rather low as revealed by electron microscopic and enzymatic controls. The pattern of distribution of the $(Ca^{2+} + Mg^{2+})$-ATPase was similar to that of the plasma membrane markers 5′-nucleotidase, $(Na^+ + K^+)$-ATPase and acetylcholinesterase and clearly different from that of the mitochondrial marker succinate dehydrogenase, or the endoplasmic reticulum marker glucose-6-phosphatase. Distinction from mitochondrial Ca^{2+}-ATPase was afforded by its insensitivity to NaN_3 and oligomycin. It differs from $(Na^+ + K^+)$-ATPase in its insensitivity to ouabain. Moreover, no difference between Ca^{2+} capture by the membrane vesicles was observed in the presence and absence of oxalate, suggesting that the $(Ca^{2+} + Mg^{2+})$-ATPase promoting it does not originate in endoplasmic reticulum [19]. (3) The $(Ca^{2+} + Mg^{2+})$-ATPase activity was very stable, even after detergent treatment of the membrane, in contrast to $(Na^+ + K^+)$-ATPase.

Previous work described the presence of the $(Ca^{2+} + Mg^{2+})$-ATPase in squid optic nerves but the percentage yield was rather small, amounting to 7% of the activity initially present in the homo-

genate [9]. In this preparation, a yield of 30% was obtained and after Nonidet P-40 treatment of the membrane the $(Ca^{2+} + Mg^{2+})$-ATPase specific activity was about 15-times higher, compared to the homogenate. However, we could not increase the ratio of $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity to the basal Mg^{2+}-ATPase activity by detergent treatment.

The dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase activity on the free calcium concentration revealed the presence of a high-affinity component and a relatively low-affinity component and this behavior was maintained after detergent treatment. The low-affinity component, previously observed in other cells [20–23], might represent a calmodulin-binding site [21] from which calmodulin is removed during the membrane isolation. An alternative explanation is that at Ca^{2+} concentrations exceeding the EGTA buffering capacity (over 1 μM), Ca · ATP would be formed and this might be hydrolyzed more rapidly than ATP [22]. Whichever the case, only the high-affinity component of the $(Ca^{2+} + Mg^{2+})$-ATPase was specifically inhibited by vanadate.

Finally, calmodulin stimulated the $(Ca^{2+} + Mg^{2+})$-ATPase activity and this finding, the inhibition of the uncoupled Ca^{2+}-pump by the calmodulin antagonist W-7 which has been observed in dialyzed squid axons (DiPolo and Beaugé, unpublished results) and the finding of endogenous calmodulin in squid optic nerves, all together suggest that the Ca^{2+}-pump could be modulated by calmodulin in vivo. However, a specific study is needed in order to support this idea.

This preparation is now used to characterize the ATP-driven Ca^{2+} uptake by plasma membrane vesicles from squid optic nerve.

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