Biochimica et Biophysica Acta, 689 (1982) 457–463
Elsevier Biomedical Press

BBA 71272

CHARACTERIZATION OF THE ($Na^+ + K^+$)-ATPase IN A MEMBRANOUS PREPARATION FROM THE OPTIC GANGLION OF THE SQUID *(LOLIGO PEALEI)*

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(Received October 13th, 1981)
(Revised manuscript received April 19th, 1982).

Key words: ($Na^+ + K^+$)-ATPase; Membrane fractionation; (Squid optic ganglion)

(1) A membrane fraction enriched in ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) was obtained from optic ganglia of the squid *(Loligo pealei)* by density gradient fractionation of membranes followed by treatment with either SDS or Brij-58. The resulting membrane had an ($Na^+ + K^+$)-ATPase specific activity of approx. 2 units/mg and was >95% ouabain-sensitive. (2) The ($Na^+ + K^+$)-ATPase had a K_m for ATP of 0.42±0.04 mM and a pH optimum of 7.0. It was inhibited by ouabain with a K_i of 0.32±0.04 μM. (3) Optimum monovalent cation concentrations were: 240 mM NaCl, 60 mM KCl, tested with NaCl + KCl = 300 mM. (4) The Mg^{2+} dependence of hydrolysis varied with the absolute ATP concentration. At 3 mM ATP, the K_m for Mg^{2+} was 0.86±0.10 mM, and at 6 mM ATP, the K_m was 1.86±0.44 mM. High levels of Mg^{2+} caused inhibition of hydrolysis. (5) The interactions of Na^+ and K^+ were examined over a range of conditions. K^+ levels caused modulations in the Na^+ dependence in the range of 1–150 mM. (6) The ($Na^+ + K^+$)-ATPase prepared from squid optic ganglion displays properties similar to those of the sodium pump in injected nerves.

Introduction

The ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) is an integral plasma membrane protein which transports sodium and potassium ions, utilizing energy derived from ATP hydrolysis [1]. The nature of the coupling between its flux and biochemical events is not understood. The giant axon of the squid *Loligo pealei* is one of the few preparations on which the flux kinetics have been obtained with reasonable accuracy. A convenient enzyme preparation would be extremely useful in identifying the biochemical correlates of these fluxes. Several groups have obtained plasma membrane preparations containing ($Na^+ + K^+$)-ATPase activity from the optic nerves of *Dosidicus gigas* [2–5], *Sepiotheutis sepiodea* [6], and *Loligo pealei* [7–8]. The present work describes the preparation of an ($Na^+ + K^+$)-ATPase-enriched membrane fraction from the optic ganglion of the squid *Loligo pealei*. Some properties of the enzyme from this source are described. Assuming that the biochemical characteristics of the ($Na^+ + K^+$)-ATPase from squid optic ganglia and nerves are the same, the squid should provide a unique system in which it is possible to examine and compare the kinetics of ion fluxes and those of the biochemical events.

A preliminary account of this work has been presented to the American Biophysical Society ((1981) Biophys. J. 33, A46).

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Methods

Tissue source. Squid (*Loligo pealei*) optic ganglia were collected at the Marine Biological Laboratory, Woods Hole, MA, during the summer of 1978, and stored at −70°C for 12 to 18 months until use.

Preparation of microsomes. Microsomes were prepared by the method of Jørgensen [9]. Tissue from 4–6 squid (1.0–2.0 g wet wt.) was used. The final microsomal pellet was suspended in 2 mM Na_2EDTA, 50 mM imidazole (pH 7.0) to a final protein concentration of 5 mg/ml, and frozen at 0°C.

Membrane fractionation. Membranes were fractionated according to the method of Chacko et al. [10], with the following modification: the final discontinuous sucrose gradient was 9 ml each of 40%, 35%, 30%, and 20% sucrose (w/v) in 5 mM Tris (pH 7.5), spun in an SW 27 rotor at $83000 \times g$ for 90 min. Bands were collected at the 20–30% and 30–35% interfaces. The 35% layer plus the band at the 35–40% interface were combined. Each band was diluted to 30 ml with 5 mM Tris pH 7.5 and spun for 60 min at $83000 \times g$. Pellets were diluted to 5 mg/ml protein with 2 mM Na_2EDTA, 50 mM imidazole (pH 7.0).

Detergent treatment. The enzyme fraction (1.0 mg/ml for SDS and urea incubations, 2.5 mg/ml for Brij-58 incubation) was suspended in buffer (2 mM Na_2EDTA, 50 mM imidazole (pH 7.0)) containing 3 mM ATP. Detergent or urea was added very slowly, while stirring. Incubation was for 30 min at 22°C. The mixture was then layered on 9 ml of 20% sucrose (w/v) in 5 mM Tris (pH 7.5) and spun at $106000 \times g$ for 60 min. The pellet was suspended in a minimum volume of buffer.

Standard $(Na^+ + K^+)$-ATPase assay. Assays were done in 0.5 ml of medium containing 240 mM NaCl, 60 mM KCl, 3 mM $MgCl_2$, 3 mM Tris-ATP and 100 mM Tris (pH 7.3). Assay temperature was 37°C (the ouabain-sensitive hydrolysis rate at 37°C was twice the rate at 25°C; the hydrolysis rate at 37°C was independent of incubation time). The total ionic strength was 400 mM, as in artificial seawater. (The enzyme's activity was higher in low ionic strength mammalian medium.) Reactions were begun with addition of enzyme, and halted with trichloroacetic acid. Control tubes contained 1 mM ouabain. Inorganic phosphate was measured by the method of Fiske and SubbaRow [11]. Modifications of the standard assay conditions are described in the figure legends.

Protein assay. Protein was determined by the method of Lowry as modified for membrane proteins by Markwell et al. [12].

Abbreviations. Specific activity is reported as units/mg which represents μmol of orthophosphate released per min per mg protein.

Results

Membrane preparation

Initial membrane fractions were prepared from squid optic ganglia by the method of Jørgensen [9]. The microsomes obtained had a specific ouabain-sensitive activity of 0.9 ± 0.1 units/mg (S.E., $n = 4$). Since microsomes might contain considerable amounts of membranes from cells not rich in $(Na^+ + K^+)$-ATPase, the total membrane was fractionated by the method of Chacko et al. [10]. The total membrane band collected on the 42% sucrose cushion was comparable in activity, 1.0 ± 0.1 units/mg (S.E., $n = 4$), and ouabain-sensitivity to the microsomes prepared by the Jørgensen method. This band was further fractionated on a discontinuous gradient, resulting in several fractions with higher specific $(Na^+ + K^+)$-ATPase activity, Table I gives representative data for tissue components prepared by the method of Jørgensen [9] and a fractionation according to the method of Chacko et al. [10]. The fractionation does not allow a complete resolution of membrane types, but enhancement of $(Na^+ + K^+)$-ATPase activity does occur in some fractions.

The gradient fractions were treated with various detergents in an attempt to further enhance the $(Na^+ + K^+)$-ATPase specific activity by solubilizing or destroying extraneous ATPase activities. Treatment with either SDS (0.5 mg/ml) or Brij-58 (5.5 mg/ml) resulted in a preparation with increased ouabain-sensitivity (>95%), and a 60–70% enhancement of specific activity, see Table II. Higher detergent levels resulted in an inactivation of the $(Na^+ + K^+)$-ATPase.

In summary, combining a membrane fractionation procedure with SDS or Brij-58 treatment of selected fractions can result in a highly ouabain-

TABLE I

COMPARISON OF THE TWO METHODS USED TO PREPARE MEMBRANOUS ($Na^+ + K^+$)-ATPase

A. Material from four squid combined and microsomes prepared by the method of Jørgensen [9]. B. Optic ganglia from six squid homogenized and fractionated by the method of Chacko et al. [10].

Preparation	Total protein (mg)	Specific activities (units/mg)		Total ouabain-sensitive ($Na^+ + K^+$)-ATPase		
		Total ATPase	Ouabain-sensitive	μmol/min	Yield (%)	Purification factor
A. Microsomes						
Homogenate	107.5	0.6	0.4	40.0		1
Low speed pellet	41.4	1.0	0.6	24.4		
Combined supernatants	64.0	0.4	0.3	16.6	100	
High speed supernatant	35.0	0.1	0.03	1.2		
Microsomes	14.7	1.8	1.0	15.3	92	2.5
B. Membrane fractionation						
Homogenate	152.0	0.6	0.4	59.3		1
Membrane band	19.6	2.2	1.2	23.5	100	3
Gradient fractions:						
20–30% band	0.16	1.7	1.3	0.2	1	3.25
30–35% band	2.9	2.8	2.1	6.0	26	5.25
35% layer + 35–40% band	4.9	3.2	2.2	10.8	46	5.5

sensitive membranous preparation of squid brain ($Na^+ + K^+$)-ATPase with a specific activity greater than 2 units/mg, representing a 6-fold increase over the homogenate.

Characterization

A variety of kinetic parameters were determined on a microsomal preparation, to allow preliminary comparison with other ($Na^+ + K^+$)-ATPase preparations. The optimum ratio of Na^+ to K^+ was determined by fixing their sum at 300 mM and varying their concentrations reciprocally (Fig. 1A). Maximal activity was achieved with 240 mM NaCl and 60 mM KCl. The pH dependence of the ouabain-sensitive activity had an optimum centered at pH 7.0 (Fig. 1B). The K_m for ATP in the

TABLE II

COMPARISON OF THE EFFECTS OF VARIOUS DETERGENT TREATMENTS ON ($Na^+ + K^+$)-ATPase ACTIVITY

Membranes were incubated as described in Methods and the final pellet was assayed by the standard ($Na^+ + K^+$)-ATPase assay method.

Treatment	Concn.	Total hydrolysis (units/mg)	Ouabain-sensitive hydrolysis (units/mg)	% Increase in specific Activity	% Ouabain sensitivity
A. Control	–	1.55	1.20	–	77
B. Urea	1.0 M	1.84	1.24	0	67
	1.5 M	1.75	1.17	0	67
C. Brij-58	5 mg/ml	1.66	1.58	32	95
	5.5 mg/ml	2.10	2.03	69	97
D. SDS	0.1 mg/ml	1.62	1.29	8	80
	0.5 mg/ml	1.97	1.90	58	96

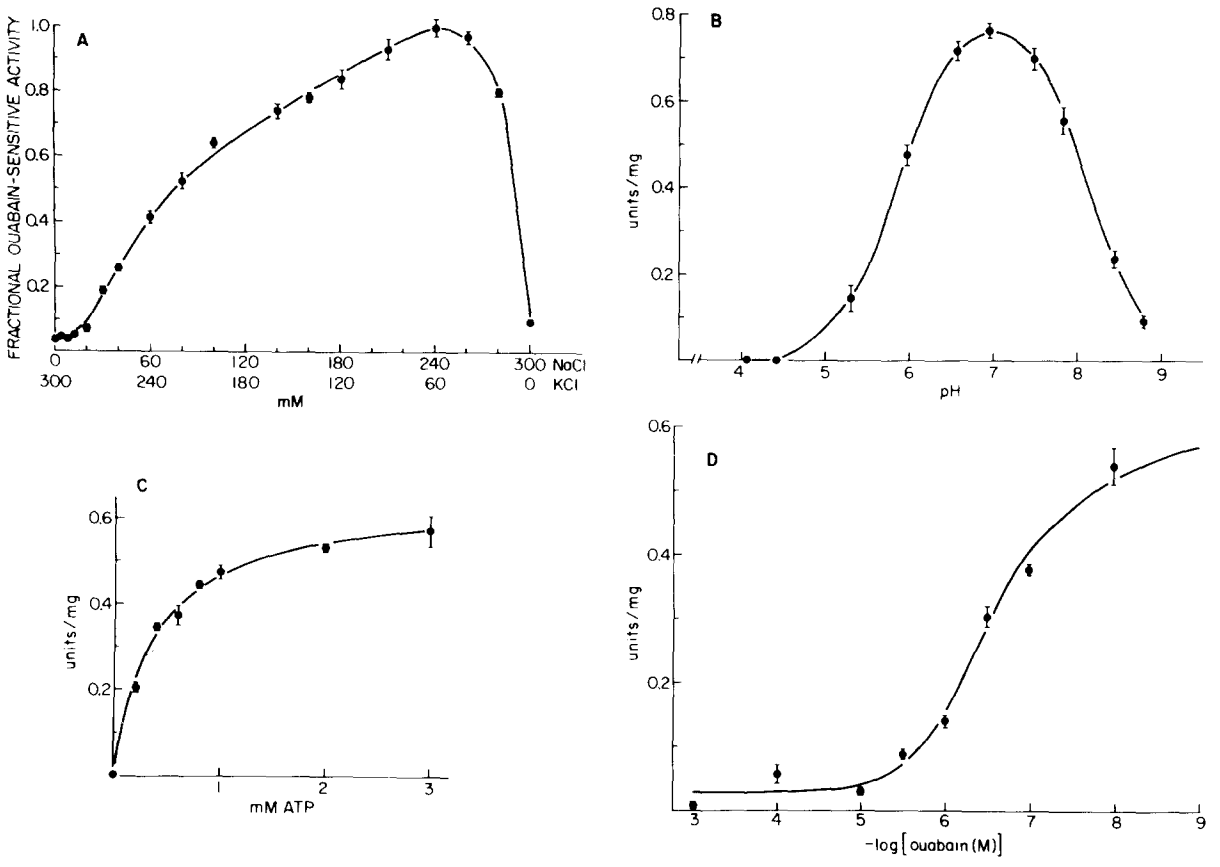

Fig. 1. Properties of $(Na^+ + K^+)$-ATPase from squid optic ganglion. (A) Skou plot. The reaction medium contained NaCl + KCl = 300 mM, 3 mM $MgCl_2$, 3 mM ATP, and 100 mM Tris (pH 7.3). Fractional ouabain-sensitive activity was calculated by setting the ouabain-sensitive activity at 240 mM NaCl + 60 mM KCl = 1.0, and calculating all other activities as fractions thereof. (B) pH dependence. The reaction medium contained 240 mM NaCl, 60 mM KCl, 3 mM each $MgCl_2$ and ATP, and 100 mM total buffer (consisting of mixtures of 0.2 M histidine, 1 M acetic acid and 1 M Tris to give desired pH at 37°C). Activity plotted is ouabain-sensitive activity in units/mg. (C) K_m for ATP. Standard assay medium (see Methods) containing variable ATP levels was used. (D) Ouabain inhibition. Enzyme was preincubated with ouabain and 1 mM ATP at 37°C for 20 min. The reaction was initiated upon addition of 2 mM ATP and halted with trichloroacetic acid. A blank without ouabain was run at each level of ouabain to allow correction for ATP breakdown during the preincubation period. Activity plotted is the activity in the presence of a given level of ouabain minus the activity at 10^{-3} M ouabain.

presence of 240 mM NaCl, 60 mM KCl, and 3 mM $MgCl_2$ was 0.42 ± 0.04 mM (Fig. 1C).

The concentration-dependence of inhibition by ouabain was examined in a microsomal preparation, Fig. 1D. The data are compatible with the binding of a single ouabain molecule per enzymatic unit: the data were fitted by the non-linear least-squares method, to a titration curve for a single binding site; the K_i for ouabain was 0.32 ± 0.04 μM.

The effect of 0–20 mM $MgCl_2$ on ATP hydrolysis was examined at 3 and 6 mM ATP, Fig. 2A and B. Both ouabain-sensitive and ouabain-insensitive activities are shown. At 3 mM ATP, Fig. 2A, both activities rise to different maximal hydrolysis rates at approx. 10 mM $MgCl_2$. The K_m for Mg^{2+} of the ouabain-sensitive activity was 0.9 ± 0.1 mM (S.E.), while the K_m for the ouabain-insensitive activity was 1.8 ± 0.3 mM (S.E.). The ouabain-sensitive activity decreased to zero at 50 mM $MgCl_2$, but there was no inhibition of the ouabain-insensitive activity (data not shown). At 6 mM ATP, Fig. 2B, the respective K_m values were increased to 1.9 ± 0.4 mM (S.E.), and 2.8 ± 0.4 mM (S.E.). High

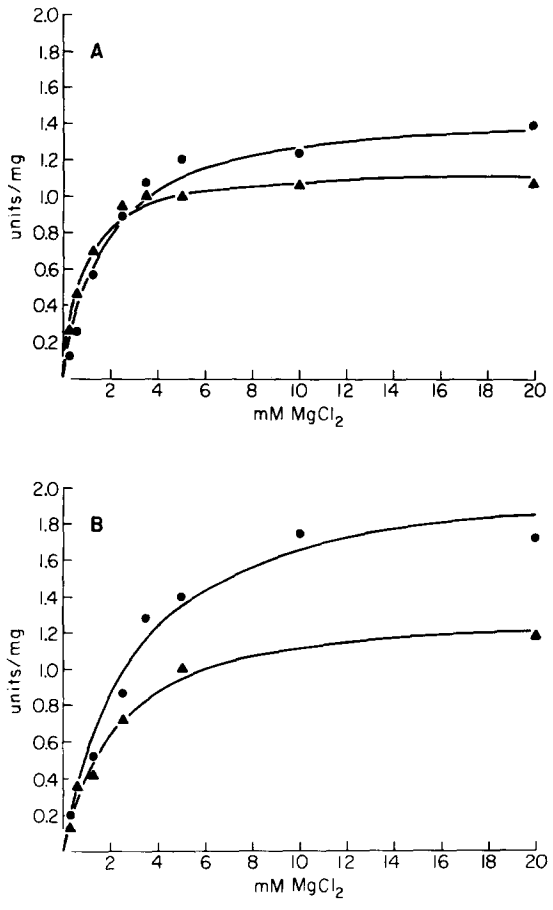

Fig. 2. Mg^{2+} dependence of ouabain-sensitive and ouabain-insensitive ATPase activities present in squid optic ganglion. The ouabain-sensitive activity (▲) was obtained by subtraction of activity seen in the presence of ouabain from the total activity. The ouabain-insensitive activity (●) is that observed in the presence of 1 mM ouabain. The medium contained 240 mM NaCl, 60 mM KCl, $MgCl_2$ + Tris (pH 7.3) = 100 mM and either (A) 3 mM ATP or (B) 6 mM ATP. The assays were performed on a microsomal preparation which was approx. 50% ouabain-sensitive.

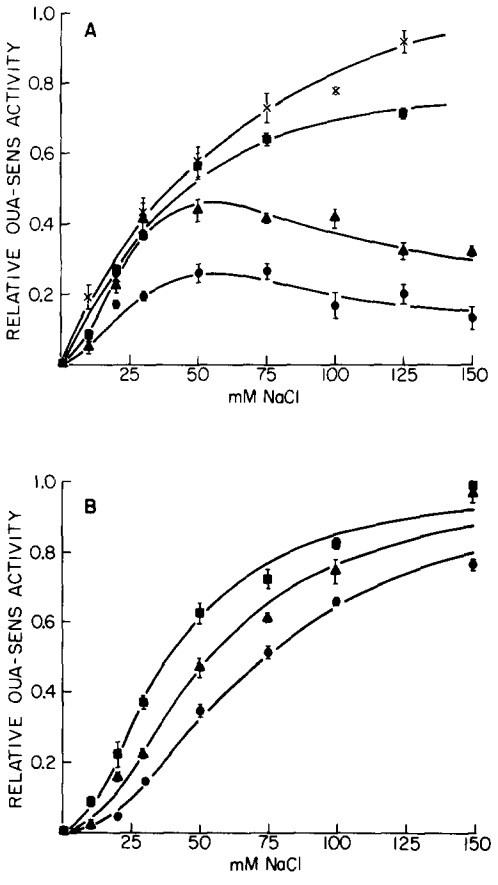

Fig. 3. Na^+-dependence of ($Na^+ + K^+$)-ATPase activity at various K^+ levels. Reaction medium contained 100 mM Tris (pH 7.3), 3 mM each of ATP and $MgCl_2$, and NaCl + KCl + Tris (pH 7.3) = 300 mM. (A) ●, 1 mM KCl; ▲, 2 mM KCl; ■, 5 mM KCl; and ×, 10 mM KCl. (B) ●, 20 mM KCl; ▲, 75 mM KCl; and ■, 150 mM KCl.

levels of Mg^{2+}, e.g. 50 mM, reduced the ouabain-sensitive activity to 53% of that at 10 mM (data not shown). This could, in part, be due to increased ionic strength.

The effect of K^+ on the Na^+-dependence of ouabain-sensitive ATP hydrolysis was examined at eight K^+ levels in the range of 1–150 mM. Na^+ was varied from 0 to 150 mM (Fig. 3A and B). At 1 and 2 mM K^+, Na^+ levels up to 50 mM stimulated ATP hydrolysis, but higher levels caused inhibition. In the range of 5–20 mM K^+, increasing Na^+ caused an increase in ouabain-sensitive hydrolysis, with no sign of inhibition at high levels of Na^+. K^+ levels in the 5–20 mM range stimulated ATP hydrolysis. At concentrations above 20 mM, K^+ caused an inhibition of ATP hydrolysis, and the curves became sigmoidal.

Discussion

Several groups have attempted to obtain plasma membrane preparations from squid nervous tissue. In most cases, ($Na^+ + K^+$)-ATPase activity was used as a plasma membrane marker, which allows

a comparison to be made with the present results.

Retinal nerves from the giant Chilean squid *Dosidicus gigas* yielded membrane preparations containing $(Na^+ + K^+)$-ATPase with a ouabain-sensitive specific activity of 0.8–1.0 units/mg, and the total ATP hydrolysis activity was 40–50% ouabain-sensitive [2–3]. Further attempts to obtain a pure plasma membrane preparation resulted in a fraction having an $(Na^+ + K^+)$-ATPase activity of 1.8 ± 4.2 units/mg [4]. A stellar nerve membrane preparation from *Dosidicus gigas* had an $(Na^+ + K^+)$-ATPase activity of 0.2 units/mg [5].

The present microsomal preparation followed by fractionation and detergent treatment (either SDS or Brij-58) resulted in a 6-fold increase in $(Na^+ + K^+)$-ATPase specific activity compared to the homogenate, with $> 95\%$ ouabain-sensitivity.

A comparison of the characteristics of the purified $(Na^+ + K^+)$-ATPase with those of the enzyme in the dialyzed or injected giant axon would allow an estimation of the viability of the preparation. The two most reliable properties are Mg^{2+} dependence and ouabain inhibition, since each acts at a specific side of the membrane, with no trans-side effects. The results in the dialyzed or injected axon are therefore comparable with results in the non-sided membrane preparation.

The effect of Mg^{2+} has been examined in injected *Loligo pealei* giant axons [14]. Optimum Mg^{2+} for maximum ouabain-dependent Na^+ extrusion was found to be 10 mM Mg^{2+}, with inhibition of efflux at higher Mg^{2+} levels. This is in agreement with the present work, in which the optimum activity was achieved between 10 and 20 mM $MgCl_2$, depending upon the absolute ATP level. Inhibition of ATP hydrolysis was observed at high Mg^{2+} levels in the membranous preparation.

The ouabain dose response curve determined on injected *Loligo forbesi* giant axons [15] is similar to the present work, the K_i in the injected axon being 0.1 μM, and that found in the present *Loligo pealei* optic ganglion preparation being 0.3 μM.

The ATP dependence of the present preparation ($K_m = 0.42$ mM), is in line with results obtained in numerous other preparations, with K_m values ranging from 0.1 to 0.5 mM [6,16–18].

A comparison of the Na^+- and K^+-dependencies of the membrane preparation with those seen in vivo is difficult. The biochemical activity observed arises from complex interactions of the ions with stimulatory and inhibitory sites. These sites have been postulated at both inner and outer membrane surfaces, and may interact under certain ionic conditions [19].

Several features of the Na^+-K^+ interactions in the membrane preparation are worth emphasizing. There appear to be three distinct phases. At low K^+ levels, there is a simulation of hydrolysis activity by low Na^+ levels, followed by an inhibition above 50 mM Na^+, which results from Na^+ competition for K^+ sites. Intermediate levels of K^+ (5–20 mM) appear to allow stimulation by Na^+ over the entire range tested. At high K^+ levels, there is a pronounced sigmoidicity to the Na^+ dependence, indicating multiple Na^+ sites (Hill coefficient 2.2) and increasing K^+ levels cause an inhibition (probably by competition of K^+ for Na^+ sites). Further work on the sidedness of ion dependencies in the dialyzed axon may yield information which will put limitations on the possible models for the interactions between Na^+ and K^+ in the non-sided preparation.

Although it is difficult to compare sided and non-sided preparations, the characteristics of the enzyme indicate that it was not damaged during isolation, since several of its properties are comparable to the in vivo situation. This preparation will allow investigations into the relationship between fluxes and biochemical events in the $(Na^+ + K^+)$-ATPase of the squid.

Acknowledgements

Dr. P. De Weer is heartily thanked for his encouragement of this undertaking and for providing the squid optic ganglia and the nonlinear least-squares fitting program. Drs. H.G. Smith and G. Kracke are thanked for helpful technical advice and Dr. C. Rovainen is thanked for comments on the manuscript. (This work was supported by NIH grant NS 11223 to P. De Weer.)

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