Lipids Associated with the (Na^+-K^+)ATPase Activity of Normal and Denervated Electric Organs of *Electrophorus electricus* (L.)

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The authors dedicate this paper in honor of Professor Carlos Chagas Filho, founder of Institute of Biophysics, on the occasion of its 50th anniversary

Cholesterol, *Electrophorus electricus*, (Na^+-K^+)ATPase, Normal and Denervated Electrocytes, Phospholipids

The effect of denervation on the lipid metabolism and on the activity of (Na^+-K^+)ATPase isoforms from the membrane fraction P_3, which corresponds to the innervated electrocyte membrane, was evaluated. On a discontinuous sucrose gradient, normal P_3 membranes exhibit a bimodal ("a" and "b" bands) distribution of the (Na^+-K^+)ATPase activity, which upon denervation changes to an unimodal ("c" band) distribution. Using these fractions, which have a higher (Na^+-K^+)ATPase activity, we characterized the lipids at the hydrophobic protein surface boundary, (i.e., the bulk lipids that surround the protein). The results confirm that these lipids consist of phospholipids and cholesterol. The quantitative composition of the phospholipids is similar for both isoform fractions obtained from the discontinuous gradient of normal membranes, with phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine representing about 90% of the total phospholipids. Sphingomyelin, phosphatidylinositol, diphosphatidylglycerol and phosphatidic acid were in the minority. However, in the single band obtained after denervation, the three major phospholipid components decreased to 70% of the total, and a significant increase in the other phospholipids and in cholesterol was observed. The high cholesterol content of the denervated fraction may confer membrane stabilization, as it is likely to cause a decrease in the membrane fluidity and consequently in the enzyme activity.

Introduction

The electrocytes of the electric organs of *Electrophorus electricus* (L.) are highly specialized cells able to produce a synchronous discharge that generates bioelectric potentials similar to those in nerve and muscle. Propagated action potentials in electrically excitable cells are known to result from transient changes in the permeability of the cell surface membrane to cations.

The (Na^+-K^+)ATPase (E.C.3.6.1.3) depends on Mg^{2+} and is essential for the maintenance of ionic gradients and cellular homeostasis. It is described as a P-type pump, and is found in the plasma membranes of almost every animal cell. It is an integral membrane protein consisting of α- and β-subunits, whose molecular weights are of the order of 100 kDa and 50 kDa, respectively (Jorgensen, 1982). The catalytic properties are confined to the α-subunits, whereas the β-subunit, a sialoglycoprotein, seems to play a role in the membrane-insertion process (Kawakami *et al.*, 1988).

The (Na^+-K^+)ATPase activity is known to be modulated by lipids in two major ways: (a) through binding of specific lipids to the hydrophilic and hydrophobic regions of the protein-lipid interface, probably stabilizing the protein conformation (Kawakami *et al.*,1985), and (b) through

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ATP, adenosine triphosphate; DPG, diphosphatidylglycerol; (Na^+-K^+)ATPase, Na^+ + K^+ - adenosine triphosphatase; P_3N, normal innervated membrane fraction; P_3D, denervated membrane fraction; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pi, inorganic phosphate; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin layer chromatography.

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the physicochemical state of the lipids, which can be more or less fluid depending on the temperature (Raison, 1973).

A microsomal membrane fraction, P_3, is obtained by differential centrifugation of homogenates of *Electrophorus electricus* (L.) main electric organ. This fraction is enriched in AChE, which serves as a marker for the postsynaptic membrane (Changeux *et al.*, 1969 and Somló *et al.*, 1977). Treatment with a solution of high ionic strength eliminates up to 85% of the AChE activity, as well as other peripheral proteins. After centrifugation of the treated fraction P_3, on a sucrose gradient, two isoforms (separate bands) with different densities and ATPase activities are obtained (Gomes Quintana *et al.*, 1992).

Denervation techniques have been used by many authors to study the relationship between nerves and related structures, as the denervated muscles undergo changes in permeability and electrical properties. Reports on alteration in biochemical parameters of plasma membranes from skeletal muscle and electric organ after denervation have appeared in the last decades (Smith and Appel, 1977; Falcato-Ribeiro *et al.*, 1980; Torres da Matta *et al.*, 1985). This article is a report on the quantitative analysis of the effect of denervation on the lipid environment of (Na^+-K^+)ATPase isoforms in the microsomal fraction.

Materials and Methods

All experiments were performed with specimens of the electric eel *Electrophorus electricus (L.)* obtained from "Museu Goeldi" (Belém-Pará) and kept at the aquarium facilities of the Instituto de Biofísica Carlos Chagas Filho, in Rio de Janeiro.

Preparation of electrocyte membranes

The membrane fractions rich in (Na^+-K^+)ATPase and AChE were obtained by differential centrifugation of homogenates of the electric organ of *Electrophorus electricus* (L.) and prepared as described by Somló *et al.* (1977). Usually 200 g of freshly minced tissue were suspended in 300 ml of 0.25 M sucrose solution containing 0.1 M NaI, 1 mM β-mercaptoethanol, pH 7.6, and homogenized for 1 min at 4°C in a Virtis apparatus at 80% of its maximal speed. The homogenate was left overnight, at 4°C, with gentle stirring, and centrifuged at $5000\times g_{av}$ for 20 min at 4°C. The supernatant was discarded and the residue was resuspended in 200 ml of 0.05 M Tris-HCl containing 0.05 M NaI, pH 7.6, and again homogenized at 4°C for two periods of 30 sec, using a Waring blendor, at 80% of its maximal speed. This homogenate was left overnight at 4°C with gentle stirring and then centrifuged for 30 min as described above. The residue (P_1) was discarded and the supernatant (S_1), after filtering through two layers of cheesecloth, was centrifuged at medium speed ($10{,}000\times g_{av}$) at the same temperature, for 30 min. The resulting supernatant (S_2), was then centrifuged at high speed ($100{,}000\times g_{av}$) for 120 min at 4°C. The supernatant was discarded and the pellets, P_2 and P_3, obtained from the last two centrifugations were suspended in small volumes of 0.05 M Tris-HCl, containing 0.05 M NaI, pH 7.6, and were gently homogenized in a Potter apparatus. The P_2 fractions, containing membranes from the electrocyte anterior (non-innervated) face, and the P_3 fraction with membranes from the posterior (innervated) face of the electric organ, consist of inside-in vesicles, (de Souza *et al.*, 1979). These fractions were transferred to small flasks and stored at -196°C. Individual samples were thawed only once.

Denervation

The surgical procedure for denervation consists of a longitudinal incision, about 10 cm long, on the dorsolateral anterior part of the fish, with the animal anesthetized under optimum conditions for breathing and metabolic activities. The incision exposes the midline nerve and also the nerves that innervate the electric organ coming from the spinal cord. About 20 nerves were sectioned through the muscle and 0.5 cm of each nerve was cut out in order to avoid regeneration. One side of the main electric organ was subjected to denervation and the contralateral side was used as a control. After 30 days, the animals were anesthetized and then killed by decapitation. Denervated and normal main electric organs were dissected from recently killed eels; these organs were removed, homogenized and used for membrane preparations.

Removal of AChE enzyme

Membrane fractions from normal (P_3N) and denervated (P_3D) organs were treated with high

ionic strenght as described by Rosenberry (1975) to remove AChE, a peripheral membrane enzyme, reducing protein contaminants in the postsynaptic membrane fraction. Briefly, membranes were suspended in 1M sodium chloride, kept on ice for 120 min and then centrifuged at 100,000×g_{av} for 30 min at 4°C. The membranes were washed twice with 50 mM Tris-HCl, pH 7.6 buffer, to eliminate the salt.

Sucrose gradient fractionation

AChE-depleted membrane fractions (P_3) from the normal and denervated electric organs were further subfractionated on a four-step discontinuous sucrose gradient (15, 25, 35 and 45%), according to Gomes Quintana *et al.* (1992), with modifications. Aliquots of P_3 containing approximately 10 mg of protein were applied to the sucrose gradient, and centrifuged at 150,000×g_{av} for 120 min at 4°C. Fractions were collected according to the appearance of the bands, from top to bottom.

Enzyme assays

(a) (Na^+-K^+)ATPase activity was measured at pH 7.6, in the following medium: 2.5 mM ATP, 3.25 mM $MgCl_2$, 0.2 mM EDTA, 24 mM KCl, 72 mM NaCl, 160 mM Tris-HCl pH 7.2. Membrane fractions were added to a final concentration of about 20 μg protein per milliliter and incubations carried out at 37°C for 15 min. The reaction was started by the addition of ATP and was stopped with 5% trichloroacetic acid. Enzyme blanks were included in each experiment and aliquots were used for inorganic phosphate (Pi) estimation by the method of Fiske and SubbaRow (1925). The specific activities were expressed as the amount of Pi liberated per mg protein per hour. Protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Membranes were also exposed for 10 min at room temperature to the inhibitor ouabain (0.4 mM), in the same medium as for (Na^+-K^+)ATPase, followed by addition of substrate, incubation at 37°C and estimation of Pi liberated by hydrolysis as described above.

(b) Acetylcholinesterase (AChE) activity was assayed according to Hassón and Liepin (1963). The enzyme activity was measured at pH 8.0. The acetic acid released from a medium containing 0.28 M ACh in 0.1 M sodium acetate and 0.2 M $MgCl_2$ was titrated with 0.1 M NaOH, after the enzyme addition. A Beckman Century SS-1 pH-meter was used and 0.1 M NaOH was delivered from an micrometer syringe (AGLA) with continuous stirring, at 25°C. Readings were taken at 1-min intervals over a 4-min period. Specific activity is defined as μmol of acetylcholine hydrolysed per mg protein per hour.

Electrophoresis

Membrane fractions P_3 (20 μg protein) were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis, performed according to Laemmli (1970), before and after the treatment for removal of AChE. Homogeneous gels containing 10% polyacrylamide were used and proteins were stained with 0.1% Coomassie Brilliant Blue G-250.

The following proteins served as Mr markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) (Sigma Chemical Co. MS-SDS-200 kit).

Lipid Extraction

Total lipids were extracted with a mixture of methanol-chloroform (MeOH:$CHCl_3$ 2:1, v/v), according to Kates (1988). The solvent mixture (3.75 ml) was added to 1 ml of a suspension of the electric organ fraction in sucrose, containing up to 50 mg dry weight of the membrane fraction, and shaken intermittently for 2 h in a 15-ml glass-stoppered centrifuge tube at room temperature. After centrifugation the supernatant was transferred to another glass-stoppered centrifuge tube using a Pasteur pipet. The residue was then suspended in 4.75 ml of MeOH:$CHCl_3$-H_2O (2:1:0.8, v/v), and the mixture was shaken and centrifuged. The combined extracts were diluted with 2.5 ml each of chloroform and water and centrifuged. The lower chloroform phase was withdrawn, diluted with an equal volume of benzene, centrifuged to clear and brought to dryness under a stream of nitrogen at 30°C. The residue was immediately dissolved in chloroform and made up to a known volume.

Lipid Fractionation

The method of Rouser *et al.* (1967) for lipid fractionation, using a silicic acid column, was adapted as follows: the total lipid extract in chloroform (2.0 ml) was mixed with 300 mg previously activated silicic acid (100–150 mesh, Sigma Chemical Co.) in a 15 ml conical centrifuge tube. To this mixture was added 5.0 ml of chloroform and the tube vigorously shaken in a Vortex mixer for a few minutes, followed by centrifugation at $800\times g_{av}$ for 5 min at room temperature. The supernatant was saved and the procedure repeated twice. The pooled chloroform extracts, which contain neutral lipids free of polar lipids, were evaporated under a stream of nitrogen and the neutral lipids thus obtained were measured gravimetrically.

The silicic acid containing the polar lipids was washed three times with 5.0 ml of acetone according to the procedure described above, to elute glycolipids. However, based on analytical thin layer chromatography (TLC), there were no glycolipids in our preparation.

The remaining fraction, attached to the silicic acid consisted of phospholipids and was extracted by three 5.0 ml methanol washes, following the same procedure as stated above. The methanol eluates were pooled and treated as described for the neutral lipids. All the fractions were made up to a final concentration of 10 mg/ml with chloroform, and their homogeneity and purity checked by analytical TLC.

Total lipid and cholesterol analysis

Total lipid and cholesterol were quantitated according to the procedures of Zollner and Kirsch (1973) and Abell *et al.* (1974), respectively.

Thin Layer Chromatography

Individual phospholipids were obtained through preparative thin layer chromatography (Kates, 1988) as follows: glass plates (20 x 20 cm) were coated with 0.4 mm of a slurry of silica gel G (Merck, Darmstadt) - 50 g in 120 ml of a solution of 0.01% Na_2CO_3 and allowed to dry overnight. Prior to use, they were activated at 120°C for 1 h. Samples of about 35–45 μg of the phospholipid fraction from silicic acid treatment were applied and the bidimensional chromatogram was run in a solvent system consisting of chloroform:methanol:ammonium hydroxide 28% (65:35:7.5, v/v) in the first direction and chloroform:methanol:acetone:acetic acid: water (10:2:4:2:1. v/v) in the second direction. The lipid spots were visualized by exposure to iodine vapor and marked.

The plates were placed in an oven at 60° C for the removal of the iodine and each spot was scraped from the plate, concentrated to 5 mg/ml and co-chromatographed with PL standards by unidimensional analytical TLC using choroform-methanol-water (65:35:4, v/v). Each PL was identified by comparing the Rf with the appropriate standard, as well as by specific staining with reagents for functional groups (Kates, 1988).

The amount of each phospholipid was determined by measuring of inorganic phosphorus (Bartlett, 1959) on samples scraped from TLC plates. Random areas of silica gel were taken off the plate to serve as blanks. For total phosphorus analysis, an aliquot of the original extract was dried and quantified by Bartlett's method.

Results

Removal of AChE

The electric organ from elasmobranch fish consists of cells (electrocytes) that are specialized for generation of strong electric potential. These electrocytes translate chemical signals from the neurotransmitter acetylcholine into an electric response (Rosenberry, 1975). The P_3 fraction from the innervated face of the electrocyte contains

Table I. Acetylcholinesterase activities in subcellular membrane from normal innervated (P_3N) and denervated (P_3D) fractions before and after treatment with 1M NaCl.

	Acetylcholinesterase			
	Original		Treated	
Membrane fraction	Specific activity	Total units	Specific activity	Total units
P_3N	17,000	257,040	3,200	21,568
P_3D	11,587	103,124	1,825	10,950

AChE from P_3N (normal innervated membrane fraction) and P_3D (denervated membrane fraction) were analysed as described in Materials and Methods. The AChE specific activity is given in μmol of ACh hydrolysed.mg^{-1} protein.h^{-1}. Values are the average of three experiments; SD ± 10%.

chemically or electrically excitable membranes and presents a high AChE activity (Couceiro and Freire, 1953). We have eliminated 85% of the AChE activity with treatment of the P_3N and P_3D membrane fractions, as described in Methods. To confirm this removal we measured the AChE activity (Table I).

Fractionation of the innervated membrane fraction on a sucrose gradient

When the fraction containing AChE-depleted (P_3N) membranes was applied to a discontinuous sucrose gradient, the (Na^+-K^+)ATPase activity was recovered in two bands, as described by Gomes Quintana *et al.* (1992). They were recovered at sucrose solution densities of 1.146 (band "a") and 1.186 g/ml (band "b"), with a specific activity of 123.6 μmol Pi.mg^{-1} protein.h^{-1} for band "a" and 89.9 μmol Pi.mg^{-1} protein. h^{-1} for band "b".

The AChE-depleted (P_3D) membrane fraction exhibited a unimodal distribution of (Na^+-K^+)ATPase. This unique band, which appeared at the same relative position as band "a" from the normal fraction, was recovered at a sucrose solution density of 1.137 g/ml (band "c") and had a specific activity of 74.6 μmol Pi.mg^{-1} protein.h^{-1}.

The activity profiles of the bands obtained from the sucrose gradient are depicted in Fig. 1. Fig. 1 (A) shows the distribution of protein concentration, (Na^+-K^+)ATPase and AChE activities from AChE-depleted (P_3D) membrane fractions and Fig. 1 (B) shows the AChE-depleted (P_3D) membrane fractions. In both cases, the AChE activity appeared at about the same position,as reported previously (Gomes Quintana *et al.*,1992) except with lower activity, because we had already removed the majority of the AChE activity from these fractions.

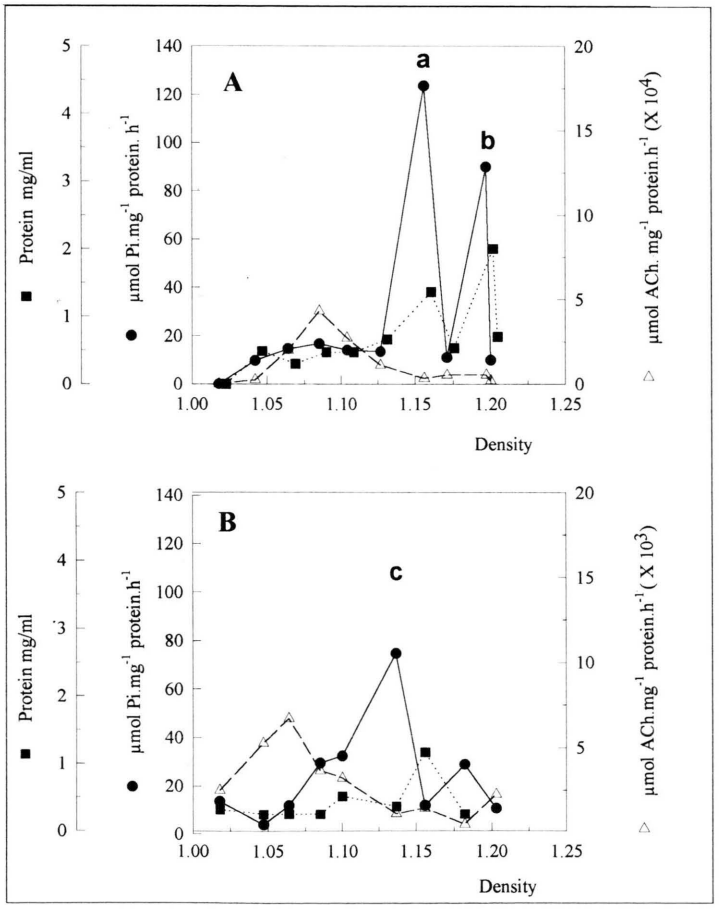

Fig. 1. Distribution of the (Na^+-K^+)ATPase (-●-●-) and AChE (-Δ-Δ-) specific activities from a discontinuous sucrose gradient of AChE-depleted P_3 membrane fractions from normal **(A)** and denervated **(B)** electrocytes. After the centrifugation, 9 fractions were collected from top to botton and analysed as indicated in Materials and Methods. Protein concentration is also indicated (-■-■-). Band "a" is obtained from the fraction that contains AChE-depleted P_3N membranes, and is recovered at a sucrose density of 1.146 g/ml. Band "b" is also obtained from the fraction that contains the AChE-depleted P_3N membranes, and is recovered at a sucrose density of 1.186 g/ml. Band "c" is the only band obtained from the fraction that contains the AChE-depleted P_3D membranes, and is recovered at a sucrose density of 1.137 g/ml.

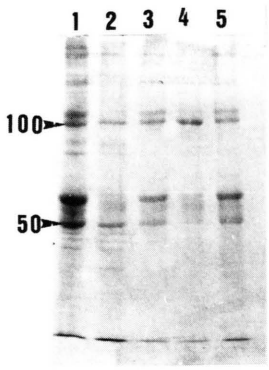

Fig. 2. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis of proteins from the normal (Lane 1) and denervated (Lane 2) AChE-depleted P_3 membrane fractions. Lane 3: band "a" from from normal membranes; Lane 4: band "c" from denervated membranes and Lane 5: band "b" from normal membranes. The arrows indicate proteins of apparent molecular weight 100 kDa and 50 kDa, which correspond respectively to the α and β-subunits of the (Na^+-K^+)ATPase. The gel was stained with 0.1% Coomassie blue as indicated in "Materials and Methods".

Fig. 2 shows a polyacrylamide SDS gel electrophoresis pattern of the proteins of AChE-depleted P_3-membrane fractions. Lane 1 presents the protein pattern before and lane 2 after the denervation. Lanes 3, 4 and 5 present the protein patterns from "a", "c" and "b" bands, respectively. The molecular weights were determined from standards of known molecular weight (not shown). The arrows indicate proteins of apparent molecular weight 100 kDa and 50 kDa, which correspond respectively to the α and β subunits of the (Na^+-K^+) ATPase.

Table II. Composition of the sucrose gradient fractions from AChE-depleted normal innervated (P_3N) and denervated (P_3D) membranes of *Electrophorus electricus*.

	Normal		Denervated
Gradient fraction	"a"	"b"	"c"
Protein	33.9	17.8	19.5
(Na^+-K^+) ATPase	123.6	89.9	74.6
Total lipid	274	98	128
Cholesterol	68.5 (25%)	17.6 (18%)	51.0 (40%)
Lipid phosphorus	6.0	4.8	4.2

The results are expressed as average of three experiments. SD ± 5%. Protein and total lipid concentration are given in mg; (Na^+-K^+) ATPase specific activity is given in μmol Pi liberated.mg^{-1} protein.h^{-1}. Cholesterol is presented in mg and under brackets in percentage of total lipids. Lipid phosphorus determined by phosphorus analysis, is presented in mg of Pi.

Determination of lipids associated with (Na^+-K^+)ATPase

Table II shows the values obtained for lipid composition, including total lipid, phospholipids and cholesterol for each of the 3 bands that exhibited (Na^+-K^+)ATPase activity after sucrose gradient centrifugation. Cholesterol comprises respectively, 25% and 18% of the total lipids from "a" and "b" bands (normal P_3- membrane fraction) but, with the denervation it increases to 40% for the "c" band.

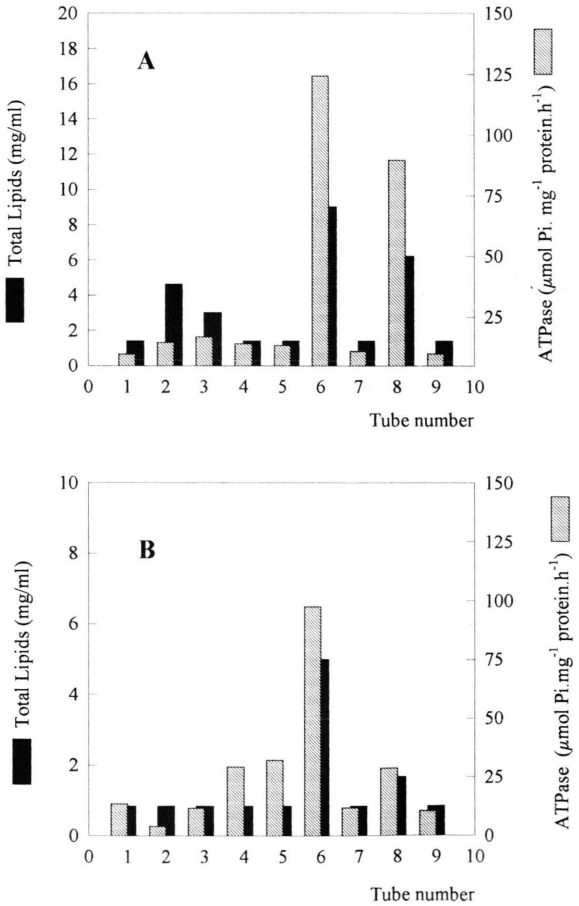

Fig. 3. Comparison of distribution of total lipid concentration and (Na^+-K^+)ATPase activity from the 9 fractions obtained by discontinuous density gradient centrifugation, collected from top to botton of the AChE-depleted P_3N **(A)** and P_3D **(B)** membrane fractions (n= 3). The experimental conditions are described under "Materials and Methods".

Distribution of total lipids and the (Na^+-K^+)ATPase activity

To confirm whether the ATPase activity is associated with a higher lipid concentration, we extracted and quantified the total lipid of all tubes obtained from the sucrose gradient. The results are depicted in Fig. 3, which shows the total lipid concentration and the (Na^+-K^+)ATPase activity from AChE-depleted P_3N membranes (A) and P_3D membranes (B). The bands with higher enzyme activity are associated with a higher lipid concentration.

Isolation of phospholipids into classes

Phospholipids were isolated by two-dimensional TLC. Each spot was characterized and its phosphorus content determined. Results are reported in Table III. We can observe that modifications occurred in the denervated fraction ("c" band) with regard to distribution among the phospholipid classes, when compared to the distribution in the "a" band of the normal fraction, which appeared in the same relative position.

Table III. Phospholipid content and composition of fractions which contains AChE-depleted normal innervated (P_3N) and denervated (P_3D) membranes.

	Membrane fraction P_3		
	Normal		Denervated
Gradient fraction	"a"	"b"	"c"
	Phospholipid content µg Pi/mg protein		
	30	16	32
Phospholipid	Phospholipid composition % of total phosphorus		
PC	58.90±3.8	56.36±0.7	43.50±0.76
PE	32.26±3.1	28.80±1.3	24.00±2.02
PS	7.87±1.8	7.13±1.4	2.76±0.15
SM	3.96±0.06	3.86±1.6	5.63±4.02
PI	2.29±0.47	3.03±1.17	3.05±0.76
DPG	1.12±0.30	2.28±0.34	4.30±0.44
PA	0.71±0.23	1.43±0.17	4.97±0.35

The results are expressed as averages with standard errors based on triplicate determinations in normal membranes and duplicates in denervated membranes. Phosphorus content was measured according to Bartlett's procedure. There was always a small percentage (± 10%) of lipid phosphorus with the same mobility as the solvent front (not computed), especially in the fraction obtained after denervation.

Discussion

Under normal physiological conditions, (Na^+-K^+) ATPase is embedded in a bilayer of membrane lipids, with protein regions sticking out of the lipid and into the aqueous medium on each side of the membrane. The enzyme activity is dependent on phospholipids that surround the enzyme; the binding of specific lipids takes place in a cooperative process (Ottolenghi, 1979), and this association changes with the state of the lipid environment (Kimelberg and Papahadjopoulos, 1974).

In this study, we have utilized the P_3 membrane fractions, obtained by differential centrifugation of electric organ homogenates. Based on AChE activity, most of the peripheral membrane proteins were eliminated by treatment with a solution of high ionic strength. We separated these membrane fractions further on a discontinuous sucrose gradient in a procedure that originated in this laboratory (Gomes Quintana *et al.*, 1992). As described, the AChE-depleted (P_3N) membrane fraction exhibits a bimodal ("a" and "b" band) distribution of the (Na^+-K^+)ATPase activity, which upon denervation changes to an unimodal ("c" band) distribution.

Lipids associated with the enzyme isoforms after denervation were determinated in microsomal P_3 membrane fractions, which are derived from the innervated face of the electrocyte membrane. In previous reports we have described metabolic changes in glycolytic (Torres da Matta *et al.*, 1985) and lipid metabolism (Quintana *et al.*, 1988) that occur upon electric organ denervation. More recently (Gomes Quintana *et al.*, 1992), we showed that denervation causes changes in the membrane (Na^+-K^+)ATPase activity, probably reflecting the readjustment of the different metabolic pathways to maintain cellular function in the denervated electric organ. In this work, after 30 days of denervation, we observed a decreased level of (Na^+-K^+)ATPase activity and also a modification in the concentration of the lipids associated with the protein. These changes are in agreement with reports by other authors, (Glynn, 1985 and Carruthers and Melchior, 1986), who state that the enzyme activity depends directly on the lipid bilayer. It is also possible that the differences in activities we have observed may be influenced by unspecific protein contaminants.

In a study of the (Na^+-K^+) ATPase from a microsomal fraction of canine kidney, Hayashi *et al.*, (1977) also obtained two peaks of this enzyme, which were attributed to different concentrations of the lipids. With the bands that we obtained by sucrose gradient centrifugation we observed that only the "a" and "b" bands from the AChE-depleted (P_3N) membrane fraction (Fig. 3A) and the "c" band from the AChE-depleted (P_3D) membrane fraction (Fig. 3B) had an increased amount of total lipid. We suggest that this finding might serve as a way to localize the (Na^+-K^+)ATPase enzyme among the other fractions.

As the purpose of this study was to characterize the lipids at the boundary of the (Na^+-K^+)ATPase, we extracted, separated and identified lipids from the fraction with ATPase activity. The results demonstrated that they are mainly constituted of phospholipids and cholesterol (Table II). As shown in Table II, the lipid-protein ratio in the bands with ATPase activity, was approximately 8:1 for the "a" band, 4:1 for the "b" band (normal fractions) and 7:1 for the "c" band (denervated fraction). All of these fractions were very rich in cholesterol, especially the membrane fraction obtained from the denervated electric organ.

We suggest that this high cholesterol concentration contributes to membrane stabilization. Cholesterol has been shown to correlate with a decrease in membrane fluidity, which in turn causes a decrease in enzyme activity. In experiments with reconstituted (Na^+-K^+)-ATPase, Kimelberg and Papahadjopoulos (1974) observed inhibition of the enzyme by cholesterol. Cholesterol markedly enhances the orientational order and consequently the packing, of lipid acyl chains (Vist and Davis, 1990; Sankaram and Thompson, 1991). Using basolateral membranes from rabbit kidney, Yagle *et al.* (1988) demonstrated that high cholesterol levels (higher than the levels found in the native membranes) inhibit ouabain-sensitive ATP hydrolysis. On the other hand, the stimulation of phosphorylation level along with hydrolytic activity by low level of cholesterol, suggests that some amount of the cholesterol actually is required for (Na^+-K^+)ATPase function (Cornelius, 1995).

Our findings for the phospholipid content (expressed in μg lipid phosphorus/mg protein) of AChE-depleted P_3N and P_3D membrane fractions, are listed in Table III. In the normal innervated (P_3N) membranes, the content of total phospholipid from "a" band (lower density and higher activity) was increased (30 μg lipid phosphorus/mg protein), whereas the "b" band (higher density and lower activity) presented lower concentrations (16 μg lipid phosphorus/mg protein). These values are in agreement with those found by other workers for normal microsomal fractions (De Pont *et al.*, 1978). In the denervated (P_3D) membranes, the single "c" band also had an elevated content of total phospholipid (32 μg lipid phosphorus/mg protein).

The content and distribution of phospholipids in the "a" and "b" bands from the normal membrane fraction were very similar. On the other hand, in the unimodal distribution obtained after denervation, we observed a decrease percent of total lipid phosphorus recovered in the identified phospholipids. When we compare the normal "a" band with the denervated "c" band, the data presented in Table 3 indicate a somewhat decreased level of PC, PE and a significant decrease for PS, indicating that they may be required for the appropriate performance of the (Na^+-K^+)ATPase enzyme. Phospholipids that are present in the inner monolayer, or negatively charged phospholipids such as PS, seem to play a fundamental role in the activation of the (Na^+-K^+)ATPase. Kimelberg and Papahadjopoulos (1972) established a relationship between the ability of phosphatidylserine and phosphatidylglycerol vesicles to cause activation of (Na^+-K^+) ATPase, indicating a possible direct involvement of the phospholipid head groups with the active center of the enzyme. Also, the anionic phospholipids, especially PS, have been described in the literature as essential for the (Na^+-K^+)ATPase activity (Ohnishi and Kawamura, 1964).

The role of phospholipids and cholesterol in the functioning of the (Na^+-K^+)ATPase is a subject that must be studied in detail. A molecular analysis of biological membranes may eventually reveal how the hydrophobic regions of the integral protein are interfaced with the lipid bilayer.

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