

Denervation alters protein–lipid interactions in membrane fractions from electrocytes of *Electrophorus electricus* (L.)

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Abstract

Protein–lipid interactions are studied in normal and denervated electrocytes from *Electrophorus electricus* (L.). Structural modifications of the lipid micro-environment encircling integral membrane proteins in membrane fractions presenting Na⁺,K⁺-ATPase activity are investigated using ESR spectroscopy of stearic acid spin labeled at the 14th carbon (14-SASL). The microsomal fraction derived from the innervated electric organ exhibits, on a discontinuous sucrose gradient, a bimodal distribution of the Na⁺,K⁺-ATPase activity, bands *a* and *b*. Band *b* is almost absent in microsomes from the denervated organ, and band *a'*, with the same density as band *a* has lower Na⁺,K⁺-ATPase activity. Band *a'* presents a larger ratio of protein-interacting lipids than band *a*. Analysis of the lipid stoichiometry at the protein interface indicates that denervation causes at least a twofold average decrease on protein oligomerization. Physical inactivity and denervation have similar effects on protein–lipid interactions. Denervation also influences the selectivity of proteins for fatty acids. Experiments in decreasing pH conditions performed to verify the influence of stearic acid negative charge on protein interaction revealed that denervation produces loss of charge selectivity. The observed modifications on molecular interactions induced by denervation may have importance to explain modulation of enzyme activity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Electron spin resonance; Spin label; *Electrophorus electricus*; Denervation; Protein–lipid interaction; Na⁺,K⁺-ATPase

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1. Introduction

Electric organs are found in different groups of fish, including the electric eel *Electrophorus electricus* (L.). Electrocytes of the electric organ are highly specialized cells able to produce a synchronous discharge that generates bioelectric potentials similar to those in nerve and muscle. They contain membrane proteins homologous to these excitable tissues, and have been used as an appropriate model system to study membrane receptors, channels and ATPases [1]. Large amounts of Na^+ channels, acetylcholine receptor and Na^+, K^+ -ATPase are present in electrocytes, and for that reason the electric organ is frequently used as a source for these proteins.

The Na^+, K^+ -ATPase, a member of the P-type class of ATPases, is unique in that it is specifically inhibited by cardiac glycosides (e.g. ouabain), making the understanding of its mechanism both physiologically and clinically relevant. It consists of α - and β -subunits, whose molecular mass are approximately 100 and 50 kDa, respectively [2]. The catalytic properties are confined to the α -subunit, whereas the β -subunit, a sialoglycoprotein, seems to play a role in the membrane-insertion process [3]. Somló et al. [4] developed a fractionation method for the electric tissue that yields a postsynaptic membrane fraction with increased Na^+, K^+ -ATPase activity. Centrifugation of this fraction on a sucrose gradient gives two separate bands with different Na^+, K^+ -ATPase activities in the normal electrocytes [5].

Nerves regulate gene expression and control the synthesis and spatial distribution of several ion channels in different muscles. Denervation techniques have been used to study the relationship between nerves and related structures. During synapse elimination, ACh receptors disperse from the synapses and their density at subregions of neuromuscular junctions decrease [6]. Denervation produces changes of expression of proteins and alters biophysical and biochemical properties of muscles, including the electric organ of electric eels [7–10]. Denervated muscles undergo changes in permeability and electrical properties. Reports on alteration in the activity of several enzymes and on the surface of plasma membranes from

muscles and electric organ after denervation have appeared [5,11–13]. Denervation has been shown to modify the distribution of Na^+, K^+ -ATPase and cholesterol content in the bands obtained from sucrose-gradient fractionation of electrocytes of *Electrophorus electricus* (L.) [14]. The distribution of phospholipid classes is also modified upon denervation [15]. The molecular mechanisms underlying all these modifications are still unclear.

Electron spin resonance (ESR) of spin labels has shown that large integral membrane proteins are able to appreciably restrict the mobility of the lipid chains in contact with the hydrophobic surface of the protein [16,17]. Since this finding, experimental evidence has appeared in support of the notion that this interface region contains targets of processes that influence functional properties. The timescale of spin-label ESR spectroscopy (rotational correlation times from 10^{-10} to 10^{-7} s) has proven useful to study the lipid–protein interface, quantify the interacting lipids and the degree of motional restriction ([18] and references therein). The lipid stoichiometry and selectivity at the protein interface, which are related to the membrane structure and degree of oligomerization of integral proteins, can be determined [17–19].

In this paper we describe differences on protein-interacting lipids of equivalent membrane fractions exhibiting Na^+, K^+ -ATPase activity obtained from innervated and denervated main electric organs of *Electrophorus electricus* (L.). ESR of a spin-labeled fatty acid is used to probe the lipid–protein interactions in these equivalent membrane fractions. The influence of temperature is also examined. Fatty acids are usually negatively charged, but their pK values for ionization equilibrium in membranes are approximately 7 [20–22]. We take advantage of this fact to investigate the influence of the electric charge of lipid headgroups on the lipid–protein interactions by performing pH titrations.

2. Materials and methods

All experiments were performed with specimens of the electric eel *Electrophorus electricus*

(L.) obtained from ‘Museu Goeldi’ (Belém, PA, Brazil) and kept at our aquarium facilities. Stearic acid spin-labeled at the C-14 position of the fatty acyl chain (14-SASL) was a gift from Prof. A. Watts (Oxford University).

2.1. Denervation and preparation of membranes

The surgical procedure for denervation [5] consisted of a longitudinal incision approximately 10-cm long on the dorsal anterior part of the fish. After being anesthetized in cold-water containing 2.0% urethane, approximately 20 nerves were sectioned through the muscle, 0.5 cm of each nerve was cut in order to avoid regeneration. Then the incision was closed with stitches. One side of the main electric organ was subjected to denervation and the contralateral side was used as a control. After 30 days in the aquarium, the animals were anesthetized and killed. The denervated region and the corresponding contralateral normal region of the main electric organ (approx. 70 g each) were removed, homogenized and used for membrane preparations.

The microsomal membrane fraction P_3 was obtained by differential centrifugation of homogenates of the electric organ of *Electrophorus electricus* (L.) and prepared as described by Somló et al. [4], at 4°C. This fraction rich in AChE contains membranes from the electrocyte anterior (innervated) face and consists of inside-in vesicles [23]. P_3 fractions were treated with high ionic strength as described by Rosenberry [24] to remove AChE and other peripheral membrane proteins. Briefly, membranes were suspended in 1 M sodium chloride, kept on ice for 120 min and then centrifuged at $100\,000 \times g_{av}$ for 30 min at 4°C. The membranes were washed twice with 50 mM Tris-HCl buffer, pH 7.6, to eliminate the salt.

AChE-depleted P_3 membranes were sub-fractionated on a four-step discontinuous sucrose gradient (15, 25, 35 and 45%) according to Gomes-Quintana et al. [5], with modifications. Fractions were collected according to the appearance of the bands and the Na^+, K^+ -ATPase activity was mea-

sured. Membrane fractions were analyzed by SDS gel electrophoresis using a 5–20% polyacrylamide gradient. Amersham SDS molecular weight marker kit was used as protein standard. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250.

For the experiments with protein-free liposomes, lipids were extracted with a mixture of methanol-chloroform (MeOH/ $CHCl_3$ — 2:1, v/v), according to Kates [25]. Total lipids were quantified according to the procedure of Zöllner and Kirsch [26] in order to estimate lipid-protein mass ratio.

2.2. Enzyme assays

Na^+, K^+ -ATPase activity was measured at pH 7.6, 37°C according to Barriviera and Hassón-Voloch [14]. Inorganic phosphate (Pi) was measured by the method of Fiske and SubbaRow [27]. Activity of membranes exposed to the Na^+, K^+ -ATPase inhibitor ouabain (0.4 mM) was also measured. Protein concentration was estimated by the method of Lowry et al. [28] using bovine serum albumin as a standard. The specific activities are expressed as the amount of Pi liberated per mg of protein per hour.

Acetylcholinesterase (AChE) activity was assayed according to Hassón and Liepin [29]. The enzyme activity was measured at pH 8.0.

2.3. Spin-labeling of membranes

Vesicles, either those obtained from electrocytes as described above or vortex-mixed dispersions of extracted lipid, were labeled at an initial label to lipid molar ratio of 1:100. Small aliquots of an ethanol solution of 14-SASL (5 mM) were injected into dilute membrane suspensions, vortex mixed and stored overnight at 4°C. The membranes were pelleted by centrifugation ($14\,000 \times g_{av}$, 20 min) and washed to eliminate the labels in the aqueous phase. The pellets were resuspended in small volumes of 50 mM Tris-HCl buffer, pH 7.6 and transferred to 100- μ l flame-sealed glass capillaries and repelleted for ESR measurements.

2.4. ESR measurements and spectral analysis

ESR measurements, unless otherwise stated, were carried out at 7°C on a Bruker ESP300E spectrometer equipped with a Varian nitrogen flow temperature controller. The temperature was chosen to facilitate spectral deconvolution. Modulation amplitude of 0.1 mT, 12 mW microwave power and 10 mT field sweep were used. All digitized spectra were normalized to the same number of spins (same double integral) before the analysis of multi-component spectra. The component line shapes were derived by spectral subtractions, as described elsewhere [30]. Here, the narrow triplet spectrum of spin labels in the aqueous phase was subtracted from all the ESR spectra before the two-component analysis. The spectrum of extracted-lipid dispersions provided the line shape for the fluid lipid component corresponding to the bulk lipid of electrocyte membranes. The motionally restricted lineshape was obtained by subtraction of the above reference spectra, and was compared to the lineshape of extracted-lipid dispersions motionally restricted by decreasing the temperature. The fractions of each component are obtained directly from the subtractions since all the spectra refer to the same number of spins.

3. Results and discussion

3.1. Enzymatic activities of membrane fractions

In order to study integral membrane proteins, most peripheral proteins are eliminated using high ionic-strength treatment as described in Section 2. AChE is a peripheral membrane protein that terminates the acetylcholine signal within synapses between neurons and electrocytes. The high AChE activity of P_3 membrane fraction indicates that it derives from the innervated face of electrocytes. Measurements of AChE activity are presented in Table 1 and show the extent of AChE-depletion. After treatment, both normal and denervated P_3 fractions present a 10-fold reduced AChE activity. Na^+, K^+ -ATPase activity of P_3 is approximately $130 \mu\text{M Pi mg}^{-1} \text{ h}^{-1}$, and

Table 1

Acetylcholinesterase activity before and after high ionic strength treatment^a

	AChE activity ($\mu\text{M ACh mg}^{-1} \text{ min}^{-1}$)	
	Normal	Denervated
Before	390 ± 140	310 ± 160
After	42 ± 21	36 ± 17

^aAcetylcholinesterase specific activity (μmol of ACh hydrolysed per mg of protein per min) and Na^+, K^+ -ATPase specific activity (μmol of Pi liberated per mg of protein per h). Results are means from experiments with four fish. Standard deviations are shown.

constitutes approximately 10% of the activity of highly purified enzyme [31]. Treatment with high ionic strength improperly decreases Na^+, K^+ -ATPase by approximately 40%.

Sucrose gradient fractionation profiles of normal and denervated AChE-depleted P_3 membranes are different (Fig. 1a). Fractionation of AChE-depleted P_3 from normal electrocytes leads to two peaks of Na^+, K^+ -ATPase activity, designated bands *a* and *b*, recovered at sucrose densities of 1.146 and 1.186, respectively. Band *b* almost disappears in the fractionation pattern of P_3 from denervated electrocytes, while the major peak of Na^+, K^+ -ATPase activity appears at the same position as band *a*, being designated band *a'*. SDS-PAGE patterns of proteins from bands *a* and *a'* are similar (Fig. 1b). Integral proteins other than the sodium pump are present. Bands *a* and *a'* have similar lipid–protein mass ratio because of their equal densities. The values are approximately 1 (w/w), as estimated by the quantification of total lipid and protein. Typical yields of bands *a* and *a'* are approximately 4–6 mg of protein per fish.

Table 2 shows the Na^+, K^+ -ATPase specific activities for bands *a*, *b* and *a'*. Band *a'* recurrently presents lower ATPase activity than band *a*. The decreased activity of denervated membranes can be due to lower expression of the sodium pump or to modification of protein micro-environment. Many authors have found different expression of channels and pumps upon denervation [9,10,32,33], but it was observed that denervation

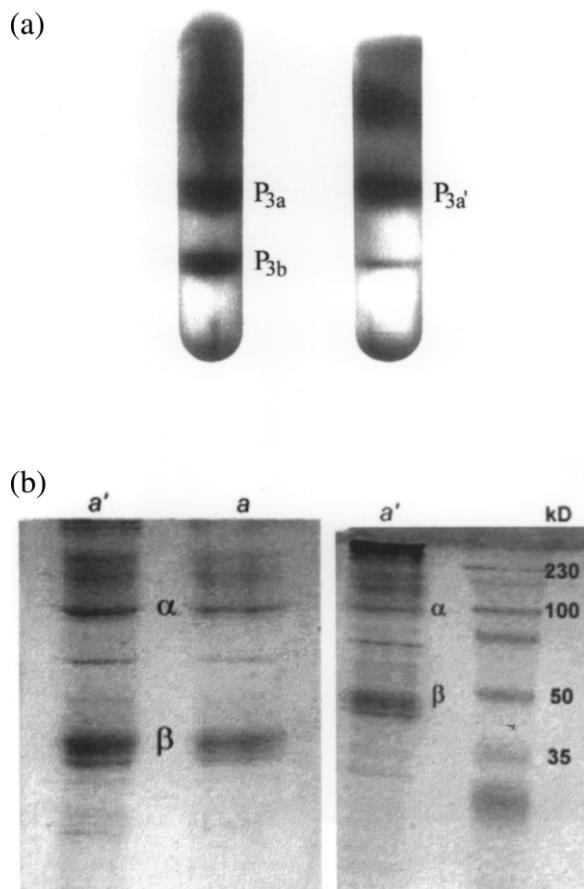


Fig. 1. (a) Negative photograph of ultracentrifuge tubes presenting the profiles of the sucrose gradient of AChE-depleted P_3 membrane fractions from normal (left) and denervated (right) electrocytes. Na^+, K^+ -ATPase activity of bands a , b and a' appears in Table 2. (b) SDS-PAGE of membrane fractions: panel on the left presents bands a and a' with similar protein composition; panel on the right presents another gel comparing band a' with molecular mass markers and indicate the positions of α and β subunits of Na^+, K^+ -ATPase.

also modifies the lipid composition [14,15]. Normal and denervated P_3 fractions have probably different distribution of Na^+, K^+ -ATPase among fractions, since band b almost disappeared in the sucrose gradient. However, bands a and a' apparently have similar protein composition because of their similar densities and electrophoretic patterns. The spin label method is used below to test for a change on protein–lipid interaction.

Table 2
 Na^+, K^+ ATPase activity of AChE-depleted membrane fractions^a

AChE-depleted P_3	Normal		Denervated
	Band a	Band b	Band a'
Na^+, K^+ -ATPase activity ($\mu\text{M Pi mg}^{-1} \text{ h}^{-1}$)	115 ± 11	86 ± 7	80 ± 7

^a Na^+, K^+ ATPase specific activity (μmol of Pi liberated per mg of protein per hour) of the sucrose gradient P_3 -fractionation bands from normal (bands a and b) and denervated (band a') electrocytes. Results are means from experiments with four fish. Standard deviations are shown.

3.2. ESR of spin-labeled lipids in membranes from normal and denervated electrocytes

The first step to analyze lipid–protein interactions by the spin label method was measuring the ESR spectrum of the spin label in pure lipid environment at different temperatures. This was performed for the spin label 14-SASL dispersed in liposomes prepared with lipids extracted from normal electrocytes. Fig. 2 shows some of the obtained spectra, all of them corresponding to the same number of spins. It shows the evolution of line-shapes and the decreasing spectral amplitudes due to increasing restriction of spin-label mobility as temperature decreases. Each spectrum consists of a single component due to labels in a uniform environment.

Integral membrane proteins restrict the mobility of the lipid chains in contact with the hydrophobic surface of the protein. ESR of spin-labeled lipids is capable of detecting this restriction. Spectral changes are analogous to those that occur on decreasing temperature [30]. This was the case of spin labeled membranes from electrocytes. The ESR spectra of 14-SASL in membrane fractions from normal, band a , and denervated, band a' , electrocytes at 7°C (Fig. 3a, 1 and 2, respectively) reveal, besides the fluid bilayer lipid population, the motionally restricted one that arises from the spin labels taking part in the belt of lipids encircling the proteins. An arrow indicates the spectral position of the low field line of the motionally restricted component. It is noted

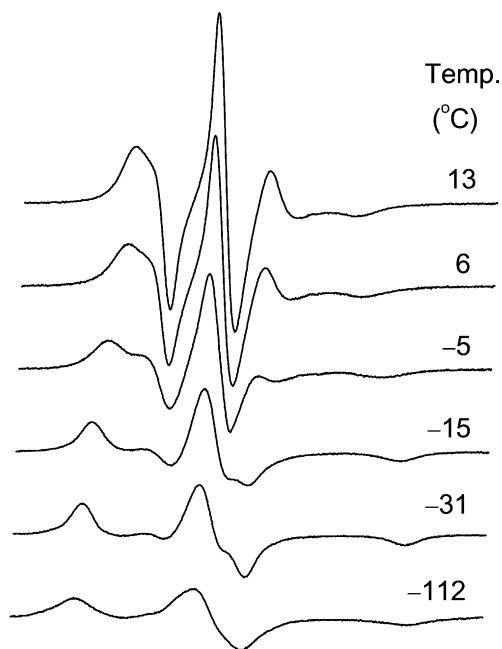


Fig. 2. ESR spectra of 14-SASL at different temperatures in dispersions of lipids extracted from AChE-depleted P_3 membrane fractions from normal electrocytes. All spectra are normalized to the same number of spins.

that this component is absent in spectrum 3 of spin labeled liposomes of extracted lipids at the same temperature, which lack membrane proteins. Nevertheless, the position of this low field line is the same as that from liposome at -31°C (cf. Fig. 2).

Comparison of the motionally restricted low field lines in spectra 1 and 2 of Fig. 3a shows that membrane fractions from denervated electrocytes present an increase in the restricted component relative to normal membranes. This indicates an increased proportion of protein-interacting fatty acids. The four fish submitted to denervation presented this property.

The fraction of motionally restricted lipids is evaluated by spectral subtraction of the bulk-lipid fluid component from the membrane spectrum. Fig. 3b presents a typical spectral analysis of the two-component spectrum of the native membranes. The results of similar analyses performed with the spectra of normal and denervated sam-

ples are listed in Table 3. Band *a* from normal electrocytes has a protein-interacting component corresponding to approximately 33% of the total species. This fraction increases to approximately 50% in band *a'*, from denervated electrocytes.

ESR measurements of spin-labeled bands *a* and *a'* were also performed at temperatures ranging from 4 to 37°C . Normal and denervated membrane fractions have analogous behavior under temperature variations. The amplitude of the

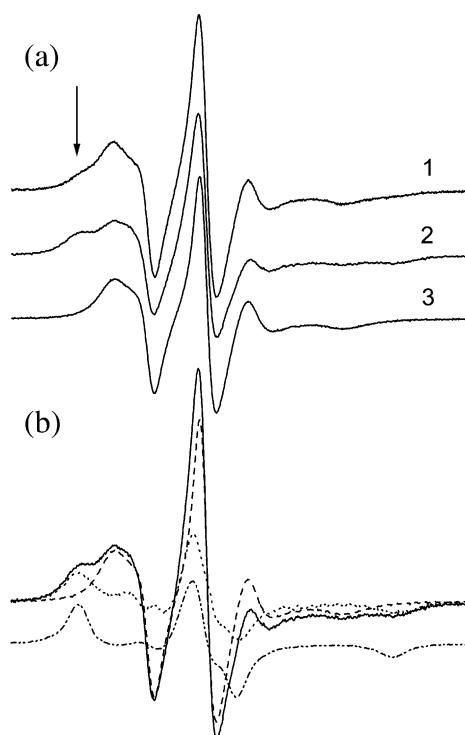


Fig. 3. (a) Experimental ESR spectra of 14-SASL spin-labeled membrane fractions from normal, band *a*, and denervated, band *a'*, electrocytes at 7°C (1 and 2, respectively). The spectrum 3 is from extracted lipids at the same temperature. The arrow indicates the position of the low field line from the protein-interacting component. (b) Typical two-component spectral analysis. Experimental spectrum obtained at 7°C of spin labeled band *a'* membranes (—). A fraction $f = 0.5$ of the normalized spectrum of liposomes of extracted lipids (----) was subtracted from the continuous spectrum yielding the protein interacting component ($\cdots$). The experimental spectrum of spin labeled liposomes of extracted lipids at -36°C (---) presents approximately the same degree of motional restriction as the dotted spectrum.

Table 3
Fraction (f) of spin-labeled fatty acids motionally restricted by the interaction with membrane proteins^a

	pH	Band	f
Normal	7.6	a	0.33 ± 0.06
Denervated		a'	0.50 ± 0.04
Normal	6.2	a	0.23
Denervated		a'	0.50

^aThe values at pH 7.6 are averages and standard deviations of measurements on membranes from four different fish. The values at pH 6.2 were obtained from the pH titrations (Fig. 4) of samples from one fish only.

bulk-lipid component increases with increasing temperature (spectra not shown), but spectral subtractions show no significant increase of the ratio between doubly integrated intensities of bulk and restricted components. This shows that the choice of temperature is not critical to perform the analysis of protein interaction with lipids and that the differences between normal and denervated membranes obtained at 7°C are also valid at higher temperatures.

Bands a and a' were also compared using samples prepared from fish kept in the aquarium for 1 year, under restricted-exercise condition. In these cases the fraction of protein-interacting lipids of band a was similar to that of band a' , suggesting that denervation and physical inactivity cause similar effects on protein–lipid interactions in electrocytes. It is worth noting that similarity between denervation and immobilization was reported by Kjeldsen et al. [43], which observed that muscle inactivity induced by denervation or by plaster immobilization of rat skeletal muscles reduces the [³H] ouabain binding-site concentration.

3.3. Number of lipid association sites on proteins

The fraction of protein-interacting lipids for a certain lipid species is determined by the intramembranous perimeter of the protein. Different lipids may have different selectivity for a given protein depending on the specificity of interactions. The selectivity for a spin-labeled lipid

in a background of unlabeled lipids can be described by a relative association constant, K_r , and the equation for the equilibrium association in an ESR experiment can be written as [18,34]:

$$\frac{(1-f)}{f} = \frac{1}{K_r} \left(\frac{N_t}{N_b} - 1 \right) \quad (1)$$

where f is the fraction of spin-labeled lipid associated with the protein, N_t is the phospholipid/protein molar ratio, N_b is the number of lipid association sites per protein, and K_r is the association constant of the spin labeled lipid relative to a non-selective lipid, usually phosphatidylcholine.

Expression (1) is used to estimate the number of association sites per protein, N_b , in normal and in denervated electrocytes. Fraction f is obtained from the ESR results. It is 0.33 for band a , and 0.50 for band a' (Table 3). The number of phospholipids per protein, N_t , is estimated as follows. The lipid–protein mass ratio is approximately 1 for both bands, as estimated above in this work. Cholesterol is 25 and 40% of the total lipid content of bands a and a' , respectively [14]. Assuming an average phospholipid molecular mass of 750 Da and a protein molecular mass of 150 kDa, the phospholipid–protein molar ratio, N_t , is 150 for band a and 120 for band a' (this difference is only due to different cholesterol content). Finally, the relative association constant of stearic acid, $K_r = 1.7$, is used after Esmann et al. [35]. It had been obtained from the restricted fraction 0.33 for 14-SASL and 0.22 for the non-selective 14-PCSL for stearic acid spin label interacting with Na⁺,K⁺-ATPase. Substituting the values of f , N_t and K_r in Eq. (1), $N_b^a = 34$ is obtained for band a , and $N_b^{a'} = 44$ for band a' ($N_b^{a'}/N_b^a = 1.3$). Both values are given per 150 kDa protein.

It is worth noting that, although the values of N_b^a and $N_b^{a'}$ depend strongly on the average molecular mass of the integral proteins, the ratio $N_b^{a'}/N_b^a$ does not. To estimate the error in this ratio, we used the similar phospholipid–protein mass ratios for normal and 30-days denervated P3 membranes measured by Gomes-Quintana et al. [15]. According to their data, phospholipid–pro-

tein mass ratio is 0.67. If this value is assumed for bands a and a' , N_t will be 134. In this case $N_b^a = 30$, $N_b^{a'} = 50$ and $N_b^{a'}/N_b^a = 1.7$ are obtained.

3.4. Aggregation / oligomerization of integral proteins

The number of associated lipids is related to aggregation of integral membrane proteins. The stoichiometry of lipids per monomer is reduced in oligomeric proteins because lipids are excluded from the monomer–monomer interfaces within an oligomer. Since N_b for band a is less than for band a' , proteins in band a have larger degree of oligomerization. Denervation decreases aggregation.

Using simple geometric considerations [18,36], it is possible to estimate the aggregation state of proteins from the lipid stoichiometry. The number of associated lipids per monomer, N_b , is related with the perimeter of the transmembranar region. For a monomeric protein the expression is:

$$N_b = \pi \left(\frac{D_p}{d_\ell} + 1 \right) \quad (2)$$

where D_p and d_ℓ are the protein and lipid diameters, respectively.

The expression for monomers in a polygonal arrangement is

$$nN_b = \pi \left(\frac{D_p}{d_\ell} + 1 \right) + n \frac{D_p}{d_\ell} \quad (3)$$

where n is the aggregation number. Expression (3), which is valid for $n > 1$, becomes clear from the sketch in Fig. 2 of Marsh and Horváth [18].

D_p/d_ℓ is unknown. To compare the aggregation state of proteins in denervated and normal bands the aggregation number $n^{a'} = 1$ is attributed to proteins in band a' and $N_b^{a'} = 44$ is used in expression (2) to obtain $D_p/d_\ell = 13$. This is substituted in expression (3) with $N_b^a = 34$, which gives $n^a = 2.1$. In the hypothesis of equal phospholipid–protein ratio ($N_b^{a'} = 50$; $N_b^a = 30$), $n^a = 3.3$. In conclusion, n^a is twice to three times

greater than $n^{a'}$, showing a two to threefold decrease in aggregation due to denervation.

It is interesting to observe that usually $D_p/d_\ell \gg 1$. In this case, it is possible to obtain an expression for estimating the increase in aggregation, n , which depends only on the ratio of associated lipids per monomer. Dividing Eq. (3) (with $N_b = N_b^n$) by Eq. (2) (with $N_b = N_b^1$) and rearranging gives:

$$\frac{1}{n} \approx \frac{N_b^n}{N_b^1} - \frac{1}{\pi} \quad (4)$$

Eq. (4) shows that the calculated decrease in aggregation due to denervation is approximately independent of the average molecular mass used in the calculations.

It is well known that denervation modifies the distribution of integral membrane proteins, e.g. AChRs and Na^+ channels in post-synaptic membranes. AChRs preferentially accumulate near the synapses in normal muscles while in denervated ones they spread over the membrane area [37,38]. The spin-label results are consistent with a dispersed distribution of membrane proteins in denervated electrocytes, and show that the dispersion alters the lipid stoichiometry of the protein interface causing at least a twofold average decrease in oligomerization.

Although the samples present low concentration of Na^+ , K^+ -ATPase active sites (less than 10% in mass of total protein, compared with highly purified samples [31]) and other integral proteins are present, the lower activity of band a' relative to band a can be connected with dissociation. Na^+ , K^+ -ATPase can function as a single heterodimer ($\alpha\beta$), but it is not yet clear whether these subunits function in cells as larger oligomers [39,40]. The fact that band a has higher ATPase activity than band a' would agree with a putative increased activity of oligomers.

3.5. pH dependence of the interaction between protein and spin-labeled lipids

Negatively charged lipids usually have in-

creased affinity for the membrane-embedded part of several integral proteins, including the Na^+, K^+ -ATPase [18,41,42]. The carboxyl polar headgroup of fatty acids is negative (ionized) in a pH 7 aqueous solution. When intercalated in neutral lipid bilayers, however, it presents ionization equilibrium with apparent pK values of approximately 7 [20–22]. It is therefore possible to neutralize the fatty acid headgroup by decreasing the pH of the buffer in which the membranes are suspended.

In order to evaluate the influence of the lipid charge on the lipid–protein interactions, ESR experiments with normal and denervated, spin labeled membranes were performed in buffers of different pH values. Some of the spectra from band *a* of normal electrocytes are presented in Fig. 4a. As the pH decreases, there is a decrease in the motionally restricted component, noticed by the reduced amplitude of the low-field line, and an increase of the bulk-lipid component. The narrow triplet spectrum of spin labels in an aqueous environment (extreme lines indicated by arrows in Fig. 4a) increases with increasing pH. This is due to the smaller lipid–buffer partition coefficient of ionized compared to neutral fatty acids.

The ESR spectrum of spin-labeled fatty acids in a pure lipid environment is also pH-dependent. For that reason, the ESR spectra of 14-SASL in dispersions of extracted lipids were obtained at each pH and used in the analysis of the two-component membrane spectra. Fig. 4b is a plot of the fraction of protein-interacting component as a function of pH. Curve 1 shows that in membranes from normal electrocytes this fraction decreases with decreasing pH, indicating that the negatively-charged fatty acids have increased affinity for integral proteins. Upon neutralization, some fatty acid molecules leave the protein annulus going to the bulk lipid environment. It is worth noting that the fraction $f = 0.23$ of protein-interacting neutral stearic acid molecules in band *a* (Table 2, pH 6.2) is similar to the value of 0.22 found by Esmann et al. [35] for the phosphatidylcholine spin label. This indicates that the neutral stearic acid is a non-selective spin label. Curve 2 displays the pH dependence for denervated electrocytes. The changes are less significant, indicat-

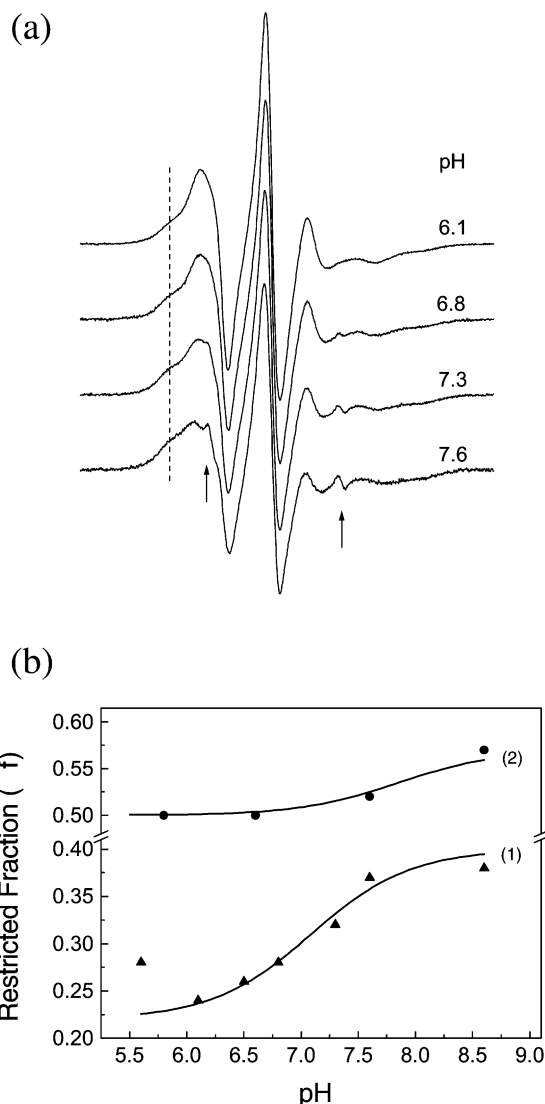


Fig. 4. (a) pH dependence of the ESR spectrum of spin-labeled membranes from normal electrocytes at 7°C. The dashed line indicates the position of the low field line from the protein-interacting component. The arrows indicate the two outermost lines of the triplet ESR spectrum from spin labels in aqueous environment. (b) Percentage of restricted component as a function of pH: (1) band *a*, normal (triangles); (2) band *a'*, denervated (circles).

ing that in this case the relative association constant is practically independent of the ionization state of the fatty acid.

Absence of charge selectivity is very unusual.

Among numerous integral membrane proteins only rhodopsin and the α -helical form of M13 phage coat protein show little selectivity [18]. However, selectivity for negatively charged stearic acid is expected to reflect not only sequence and structure at the protein annulus but also the host lipid background. Change of phospholipid composition upon denervation [14,15] associated with a probable rearrangement of amino acid residues at the protein annulus may cause both dispersion and selectivity loss.

4. Conclusions

The results of this work show evidence that protein activity, protein–lipid interactions, lipid selectivity, spatial distribution and oligomeric state of integral proteins are modified by denervation in membranes of electrocytes.

Fractionation of AChE-depleted membranes obtained from normal electrocytes of *E. electricus* (L.) on a sucrose gradient leads to two membrane bands, *a* and *b*, with Na^+, K^+ -ATPase activity. Denervated electrocytes present band *a'* at the same relative position as band *a*, with the same protein–lipid mass ratio, but almost lack band *b*. The equivalent bands *a* and *a'* have different Na^+, K^+ -ATPase activities. Specific ATPase activity is approximately 30% lower in denervated than in normal membranes.

ESR of stearic acid spin label shows that membranes from normal and denervated electrocytes, which have the same lipid–protein mass ratio, present different proportions of protein-interacting lipids. The results indicate that denervation alters the lipid stoichiometry of protein interface and causes an average decrease of integral proteins oligomerization. This is consistent with dispersion of membrane proteins out of synapses in denervated electrocytes.

It is found that membranes from denervated and normal electrocytes behave differently with respect to charge selectivity of protein–lipid interaction. The pH dependence of protein-interacting spin labeled stearic acid is remarkable for normal membranes. It shows that selectivity for negative 14-SASL is 1.7 times greater than that

for the neutral (low pH) spin label. Denervated membranes present a very weak pH dependence characterizing absence of selectivity.

It is also noticed that physical inactivity and denervation modify protein–lipid interactions in the same way.

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