Non-uniform distribution of phospholipids in $(Na^+ + K^+)$-ATPase-rich membranes from *Torpedo marmorata* electric organ evidenced by spin–spin interactions between spin-labeled phospholipids

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Received 28 July 1983

$(Na^+ + K^+)$-ATPase membranes from *Torpedo marmorata* electric organ were labeled with high concentrations of paramagnetic phospholipid analogs (up to 5 mol of spin labels for 100 mol of endogeneous phospholipids). The mobile lipid bilayer component of the complex resonance spectra obtained showed low-field linewidth broadening due to spin–spin interactions; this was used as a measure of probe concentration in this compartment. Our results show that in the lipid shell surrounding the protein, there is a considerable enrichment in negatively charged phospholipids over neutral ones.

$(Na^+ + K^+)$-ATPase *Electric organ* *Lipid–protein interaction* *Spin labeling* *Electron spin resonance* T. marmorata

1. INTRODUCTION

Spin-labeled phospholipid analogs have been widely used in membrane studies, in particular to test and quantify lipid interactions with membrane proteins [1,2]. If such interactions exist, the electron spin resonance spectrum should exhibit two components: one which is very similar to the spectrum obtained in pure lipid systems (lipid bilayer component) and another of larger splitting (motion-restricted component). Quantification of the lipid–protein interaction is often related to the amplitude of the motion-restricted component obtained by subtraction of a 'pure' mobile or immobilized component from the complex spectrum. With such an approach, interactions have been inferred in $(Na^+ + K^+)$-ATPase membranes [3] or cytochrome oxidase membranes [4]. However, an exchange process of the probe between a protein-associated state and a lipid bilayer state causes spectrum deformations [5] which in turn make subtractions difficult to achieve. We here propose a different approach: by taking advantage of linewidth broadening arising from spin–spin interactions which take place in the bilayer, we measured the concentration of the probe in the lipid bilayer component.

2. MATERIALS AND METHODS

$(Na^+ + K^+)$-ATPase-rich membranes were isolated from *Torpedo marmorata* electric organ by a method similar to that in [6], except that the sonication step was omitted. Lipids were extracted from these membranes as in [7] and liposomes obtained by sonication of these lipids under nitrogen.

Protein was estimated as in [8]. Organic phosphate concentration was determined as in [9]. Distribution of phospholipids in the different classes was checked by thin-layer chromatography [10]. Polyacrylamide gels in the presence of sodium dodecyl sulphate were performed as in [11].

The spin-labeled phospholipid analogs used here were 1-palmitoyl, 2-(3-doxyl)pentanoyl-*sn*-glycerophospholipids where the polar group was

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00145793/83/$3.00

substituted with a proton, choline, serine or ethanolamine moiety for (0,2) phosphatidic acid, (0,2) phosphatidylcholine, (0,2) phosphatidylserine or (0,2) phosphatidylethanolamine, respectively. The analogs were incorporated in membranes as follows: the desired amount in ethanol was dried down as a film in a test-tube under argon; then membranes were added and gently shaken. Incorporation of the probe was completed within 2 min. All experiments were carried out in 250 mM sucrose, 0.5 mM EDTA, 50 mM Tris–HCl (pH 7.4) buffer, at 0.8 ± 0.2°C.

ESR measurements and spectrum analysis: A Varian E 109 spectrometer fitted with a field-frequency lock was used, on line with a Tektronix 4051 computer which allows accumulation and integration of spectra. All spectra were normalized to the same integral and the height of the low-field line measured by a computer program. For Lorentzian lines the linewidth (LW) is proportional to the inverse of the square root of the line height (H). Plotting $H^{-1/2}$ *vs* probe concentration allowed us to determine H_o, the height at infinite dilution of the probe in the lipid phase. Thus, as a first approximation, linewidth broadening ($LW-LW_o$) is proportional to ($H^{-1/2}-H_o^{-1/2}$).

3. RESULTS

$(Na^+ + K^+)$-ATPase-rich membranes isolated from *T. marmorata* electric organ exhibited an enzyme-specific activity at 37°C of about 70 μmol P_i liberated $\cdot h^{-1} \cdot$ mg protein^{-1}, corresponding to previously published activities obtained with the same type of membranes [12,13]. Gel electrophoresis (fig.1) shows the purification of the $(Na^+ + K^+)$-ATPase-rich fraction over the homogenate: this fraction was devoid of acetylcholine-receptor membranes and only few proteins remained associated with $(Na^+ + K^+)$-ATPase in its membranous form; by peak integration, the enzyme represented about 70% of the proteins. As there were 0.98 ± 0.1 μmol phospholipids/mg protein, this represented 400 phospholipids/enzyme assuming 70% pure $\alpha_2\beta_2$ ATPase (288 kDa). By chromatography, phospholipids were 37% phosphatidylcholine, 12% sphingomyelin, 29% phosphatidylethanolamine, 17% phosphatidylserine and 5% phosphatidylinositol, in accord with [14].

Spectra obtained with (0,2) phospholipids are

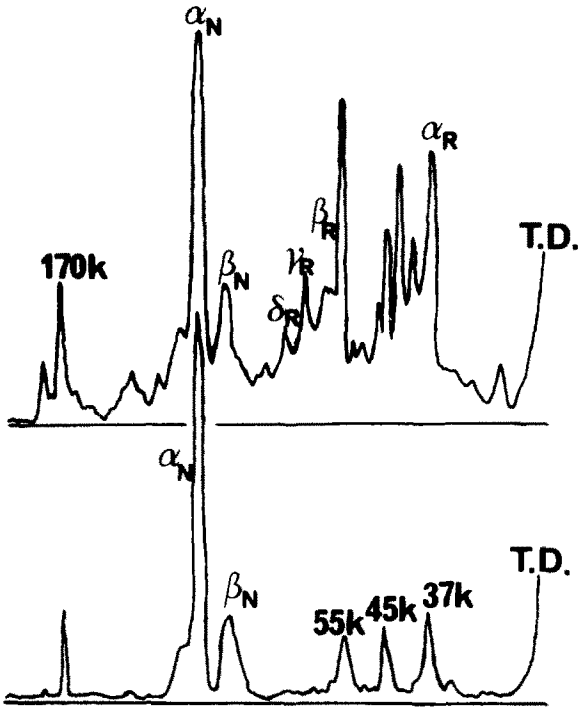

Fig.1. Scans of 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of membrane homogenate (upper trace) and $(Na^+ + K^+)$-ATPase-rich membranes (lower trace). α_N and β_N referred to $(Na^+ + K^+)$-ATPase subunits; α_R, β_R, γ_R and δ_R to acetylcholine receptor subunits; TD, tracking dye; k, kDa.

shown in fig.2. They were recorded after a 2-min incubation of membranes with the probe. It has to be stressed that the spectrum shape did not change over a 1-h period. Thus (0,2) phospholipid incorporation into the membrane was a fast phenomenon and did not require centrifugation in order to eliminate residual probe micelles or vesicles. Addition of Ni^{2+} to labeled membranes, even after a 10-h incubation, homogeneously broadened the spectrum, indicating a free access of the cation to the nitroxyde radical. This strongly suggested that a vast majority of $(Na^+ + K^+)$-ATPase membranes are leaky vesicles or fragments. In accordance, at least 95% of ATPase activity was inhibited by ouabain, and no increase of activity was detectable after gentle membrane disruption in the presence of ATP. Thus the occurrence of $(Na^+ + K^+)$-ATPase membranes mainly as leaky vesicles rules out that differences noted between various phos-

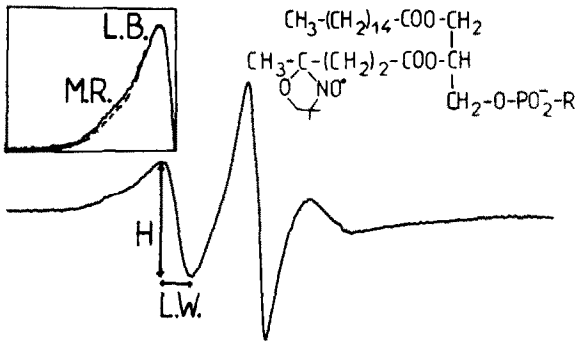

Fig.2. ESR spectrum of (0,2) phosphatidylserine in $(Na^+ + K^+)$-ATPase membranes from *Torpedo marmorata* electric organ. Inset: enlargement of low-field line obtained with (0,2) phosphatidylserine (solid line) or (0,2) phosphatidylcholine (dotted line). MR indicates the motion-restricted component which protrudes as a shoulder from the lipid bilayer component (LB). Spectra were recorded as indicated in section 2.

pholipids could be due to their asymmetric transverse membrane distribution.

The spectra in fig.2 show a low-field shoulder reflecting a motion restricted location of the probe. Comparing spectra arising from two different (0,2) phospholipids, differences in the motion-restricted components could be noted but this component remained hard to quantify. This prompted us to use another quantitative approach, which in some aspects resembles those described in [15] or [16].

We chose to measure the height and thus the linewidth of the low-field line of the mobile component because the overlap of the motion-restricted component is much less than in the medium-field line. This measurement was performed with the 4 (0,2) phospholipids incorporated either in $(Na^+ + K^+)$-ATPase membranes or in liposomes made from extracted lipids. Results are shown in fig.3: with extracted lipid vesicles, broadening was identical for all 4 probes under study (28.8 units/% of probe); with ATPase membranes, probes were distributed into two groups: phosphatidylcholine and phosphatidylethanolamine which had a broadening slightly inferior to the one noticed with liposomes (27.2 units/% of probe); phosphatidylserine and phosphatidic acid with a much lower broadening (21.8 units/% of probe). In any case, broadening was

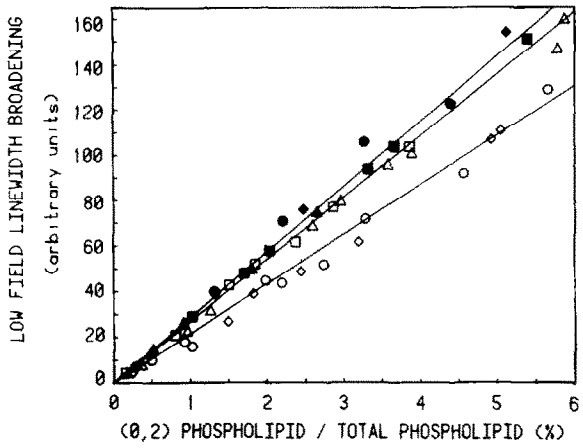

Fig.3. Low-field linewidth broadening obtained with $(Na^+ + K^+)$-ATPase membranes (open symbols) or extracted lipid vesicles (closed symbols). Broadening (arbitrary units) was expressed as a function of the percentage of total (0,2) phospholipids in membrane phospholipids. Probes were phosphatidylcholine (△, ▲), phosphatidylethanolamine (□, ■), phosphatidylserine (○, ●) and phosphatidic acid (◇, ◆). Slopes were obtained by linear regression of at least 17 different data obtained with 3 independent preparations. For the sake of clarity some of the data were omitted on this graph.

proportional to the probe concentration over a wide range, resulting in linear plots. Similar measurements were done with medium field-line leading to the same type of results (not shown).

4. DISCUSSION

Phospholipid composition of $(Na^+ + K^+)$-ATPase membranes from *Torpedo marmorata* electric organ closely resembles those described for $(Na^+ + K^+)$-ATPase membranes from other tissues (see [14]). The gross composition shows that neutral phospholipids were much more abundant than the negatively charged ones (78% *vs* 22%) in this type of preparation. Electron spin resonance experiments clearly showed that in these membranes the 4 phospholipid analogs behaved differently, contrary to what was observed with extracted lipid vesicles. In liposomes there was no significant difference between curves obtained with either one of the probes reflecting an identical distribution of phospholipids, as expected. On the other hand, when $(Na^+ + K^+)$-ATPase membranes

were studied, (0,2) phosphatidylserine and (0,2) phosphatidic acid led to curves whose slopes were lower than those arising from (0,2) phosphatidylcholine or (0,2) phosphatidylethanolamine. It is to be noted that these latter molecules exhibited a smaller broadening in membranes than in liposomes.

From fig.3 we conclude that in the ATPase membrane phosphatidylcholine and phosphatidylethanolamine distribute primarily in the bilayer. Phosphatidylserine and phosphatidic acid appear to distribute into both the bilayer and another compartment. This very likely indicates that these molecules were located around the protein surface (and thus are responsible for the motion-restricted component noticed with these membranes). If so, the lipid bilayer concentration of a given phospholipid can be calculated as:

$$[PL]_{\text{lipid bilayer}} = [PL]_{\text{total}} \times \frac{\text{slope}_M}{\text{slope}_L}$$

where:

slope_M is the slope obtained in $(Na^+ + K^+)$-ATPase membranes with the corresponding (0,2) phospholipid analog;

slope_L is the slope obtained in extracted lipid liposomes with the analogs.

Phospholipid distribution into $(Na^+ + K^+)$-ATPase membrane is summarized in table 1. We considered that sphingomyelin behaved as phosphatidylcholine as they shared the same head group. Lack of (0,2) phosphatidylinositol did not allow any quantification for this molecule. The protein surrounding lipid fraction was considerably enriched in negatively charged lipids, reaching 50%. By the subtraction method such a preferential association of negatively charged lipids with $(Na^+ + K^+)$-ATPase from dogfish salt gland or electric eel electric organ has been reported [3,17,18]. Biochemical data demonstrated that $(Na^+ + K^+)$-ATPase activity required phospholipids to function [19] and that maximum turn-over rate was maintained only if phosphatidylserine or, more specifically, phosphatidylinositol was present [14,20]. Our results with this enzyme from *Torpedo marmorata* electric organ are thus in agreement with these facts.

Table 1

Phospholipid	Mol/mol enzyme	Mol in the lipid bilayer phase	Mol trapped around the protein
Phosphatidyl-choline	148	140	8
Phosphatidyl-ethanolamine	116	110	6
Sphingomyelin	48	45	3
Phosphatidylserine	68	51	17
Phosphatidyl-inositol	20		
Total	400		

ACKNOWLEDGEMENTS

Authors are indebted to Mrs P. Hervé and Dr E. Favre for synthesis of the spin-labeled phospholipids. This work was supported by grants from the Ministère de l'Industrie et de la Recherche, the Centre National de la Recherche Scientifique (E.R.A. 690) and the Université Paris VII.

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