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PHOSPHOLIPID CHAIN IMMOBILIZATION AND STEROID ROTATIONAL IMMOBILIZATION IN ACETYLCHOLINE RECEPTOR-RICH MEMBRANES FROM *TORPEDO MARMORATA*

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1. The ESR spectra of both phosphatidylcholine and phosphatidylethanolamine spin labels reveal an immobilized lipid component ($\tau_R \gtrsim 50$ ns), in addition to a fluid component ($\tau_R \sim 1$ ns), in acetylcholine receptor-rich membranes prepared from *Torpedo marmorata* electroplax according to the method of Cohen et al. (Cohen, J.B., Weber, M., Huchet, M. and Changeux, J.-P. (1972) FEBS Lett. 26, 43–47). 2. The ESR spectra of the androstanol spin label display a component corresponding to molecules which are immobilized with respect to rotation about the long molecular axis ($\tau_R^{//} \gtrsim 50$ ns), in addition to the fluid lipid bilayer component in which the molecules are rotating rapidly about their long axes ($\tau_R^{//} \sim 1$ ns). This immobilized component is observed throughout the temperature range 2–22°C, at an approximately constant relative intensity of approx. 45% of the total, which is quantitatively the same as previously observed with fatty acid spin labels.

Introduction

In previous work [1] we have reported the existence of a two-component ESR spectrum both from fatty acid spin labels and from a steroid spin label in acetylcholine receptor-rich membranes which were prepared from the electric tissue of *Torpedo marmorata* according to the method of Cohen et al. [2]. The second component which was relatively immobilized on the conventional ESR timescale ($\tau_R \gtrsim 50$ ns), and was not present in the spectra from bilayers of the extracted membrane lipids, was attributed to a population of membrane lipid whose mobility was reduced by direct interaction with the membrane protein. Recently Rousselet et al. [3] have reported that they observe an immobilized component with fatty acid and ester spin labels, but not with a phosphatidylcholine spin label, in acetylcholine receptor-rich membranes prepared according to Sobel et al. [4]. Since the immobilized component was not observed with the phospholipid spin label, it was concluded that the immobilization of the fatty acid label arose from a specific binding to the receptor protein. In the present paper we show that an immobilized component is observed both with a phosphatidylcholine spin label and with a phosphatidylethanolamine spin label in our preparations of acetylcholine receptor-rich membranes. We also provide additional evidence on the stoichiometry of the immobilized component observed with the androstanol spin label.

Materials and Methods

Acetylcholine receptor-rich membranes were prepared from the electric organ of *Torpedo marmorata* according to Cohen et al. [2]. The specific α-toxin binding capacity, assayed by the Millipore filtration

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Abbreviations: n-SASL, n-(4′,4′-dimethyloxazolidinyl-*N*-oxy)-stearic acid; 16-PCSL (PESL), 1-acyl-2-(16-[4′,4′-dimethyloxazolidinyl-*N*-oxy]stearoyl)-*sn*-glycero-3-phosphocholine (phosphoethanolamine); ASL, 17-β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxyl.

method [5], was typically 1 nmol ^{3}H-labelled α-toxin/mg protein.

Stearic acid with the doxyl spin label group on the 16-C atom, 16-SASL, was prepared by the method of Hubbell and McConnell [6]. The corresponding phosphatidylcholine spin label, 16-PCSL (1-acyl-2-(16-[4′,4′-dimethyloxazolidinyl-*N*-oxy]stearoyl)-*sn*-glycero-3-phosphocholine) was prepared according to Boss et al. [7]. Either 1-palmitoyl-*sn*-glycero-3-phosphocholine (Fluka, Buchs, Switzerland), or egg lysophosphatidylcholine (Lipid Products, South Nutfield, U.K.) was used in this acylation. Thus in either case, the 16-PCSL had predominantly palmitoyl chains in the *sn*-1 position. The phosphatidylethanolamine spin label, 16-PESL, was prepared from the 16-PCSL spin label by head-group exchange using phospholipase D [8]. The androstanol spin label, ASL (17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxyl), was obtained from Syva, Palo Alto, CA, U.S.A.

Membranes were labelled in 9–10 ml *Torpedo* Ringer's solution [2] by adding the spin label at 0.02–0.04 mg/mg protein for 16-PESL and 16-PCSL (0.01–0.02 mg/mg protein for ASL), as a concentrated solution in ethanol. The total amount of ethanol added was less than 1% (v/v). The labelled membrane suspension was then washed twice by centrifugation and resuspended in a minimal volume of *Torpedo* Ringer's solution, for ESR measurement.

ESR measurements and spectral subtractions were performed as described previously [1,9]. The spectrum used for subtraction of the immobilized component was obtained from a lipid-depleted sample of cytochrome oxidase [9], and was recorded at a temperature which gave best correspondence with the lineshape of the immobilized component in the membrane (cf. Ref. 1). A slight adjustment was made to the scan width, in order to compensate for the differences in splitting arising from polarity effects. For further details of the spectral subtraction methods, see Ref. 18.

Results and Discussion

The spectra of the 16-PCSL and 16-PESL phospholipid spin labels in acetylcholine receptor-rich membranes are given in Fig. 1. The spectra of the phospholipid labels were complicated by an underlying spin-spin broadened component corresponding to vesicles of unincorporated label. In Fig. 1, this spin-spin broadened component has been subtracted digitally using a spectral component obtained from pure spin label vesicles. The immobilized lipid component with outer hyperfine splitting approx. 61–64 gauss is clearly seen, in addition to the fluid bilayer component, with both labels (cf. Ref. 1). Thus the immobilized lipid component is seen with phospholipid labels in addition to fatty acid labels, when using preparations and labelling conditions similar to those described previously [1]. The reason for discrepancy with the results of Rousselet et al. [3] could lie in the somewhat different preparative procedures used, and particularly in the different labelling procedures. Rousselet et al. [3] used the phosphatidylcholine exchange protein, for which rigid membranes are known to be poorer substrates, to achieve label incorporation. Thus it is possible that the label was

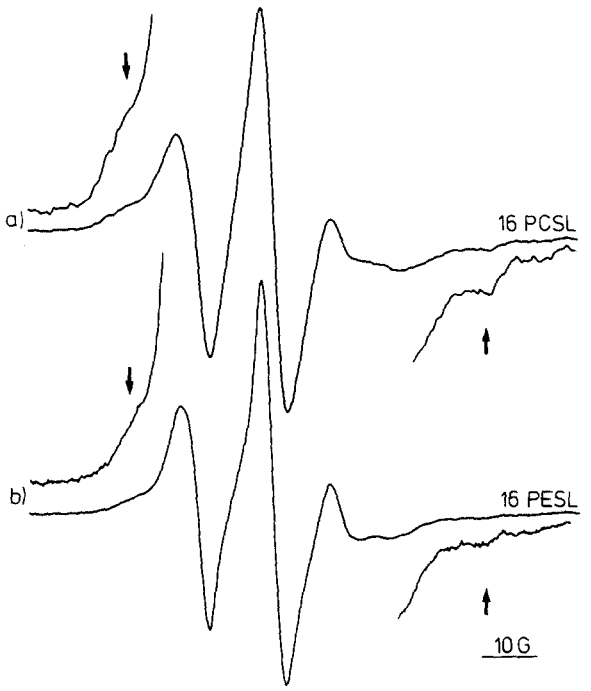

Fig. 1. ESR spectra of phospholipid spin labels in acetylcholine receptor-rich membranes from *Torpedo marmorata*. (a) 16-PCSL phosphatidylcholine spin label at −5°C. (b) 16-PESL phosphatidylethanolamine spin label at −1°C. The spin-spin broadened component from unincorporated spin label vesicles has been digitally subtracted from these spectra.

preferentially incorporated into more fluid regions of the membrane (and only into the outer monolayer).

Unlike the fatty acid and androstanol spin labels, the phospholipid spin labels are less readily incorporated into the acetylcholine receptor-rich membranes. It is not known exactly whether the spin-spin broadened component subtracted in Fig. 1 is due to unincorporated spin label vesicles trapped in the membranes, or to vesicles which have fused with the membranes but have not randomized by lateral diffusion with the membrane lipids. Since the acetylcholine receptor-rich membrane preparation is not completely homogeneous, it is also possible that there is some heterogeneity in the fusion of the spin label vesicles with the membranes. However, the significant result is that an immobilized spin label component is seen with phospholipid spin labels in the membranes and the relative intensity is comparable to that observed previously for the other labels [1]. This immobilized component is clearly seen whether the spin-spin broadened component has been subtracted out or not.

The ESR spectra of the androstanol spin label in acetylcholine receptor-rich membranes are given in Fig. 2A. An immobilized component with outer splitting of approx. 64 G is clearly seen, in addition to the fluid lipid bilayer component, at all temperatures throughout the range 8–22°C (cf. Ref. 1). The fluid lipid component in the membranes, which corresponds to labels rotating rapidly about their long axes, is clearly seen in the difference spectra of Fig. 2B in which the immobilized component has been subtracted from the membrane spectra, using the methods of Ref. 9. The fractions, f, of immobilized component in the membrane spectra, obtained by double integration of the difference spectra, are given in Table I. Also given are the fractions of fluid component, $(1-f)$, obtained by subtracting the lipid bilayer spectra from the membrane spectra, as in Ref. 1. There are uncertainties in the end-points of the subtractions at opposite extremes of the temperature range for the two methods. However, it can be seen that a consistent, essentially temperature-independent value, $f \sim 0.45$, is obtained for the fraction of immobilized androstanol label, by both methods.

The values for the fraction of immobilized steroid probe are compared in Table I with the corresponding values for the fatty acid probes, 16-SASL and 12-

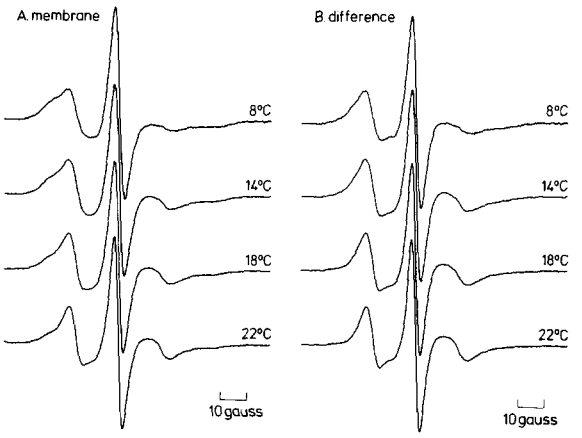

Fig. 2. ESR spectra of the androstanol, ASL, spin label in acetylcholine receptor-rich membranes from *Torpedo marmorata*. A. Membrane spectra. B. Fluid lipid bilayer component difference spectra obtained by subtracting away the immobilized component from the spectra in A.

TABLE I

FRACTION OF IMMOBILIZED LIPID COMPONENT, f, IN ACETYLCHOLINE RECEPTOR-RICH MEMBRANES FROM *TORPEDO MARMORATA* DEDUCED FROM THE ESR SPECTRA OF ANDROSTANOL AND STEARIC ACID SPIN LABELS

Error bars reflect the approximate range of indistinguishable end-points in the subtractions.

Label	T(°C)	f *	$(1-f)$ **
ASL	2	(0.45) ± 0.06	0.54 ± 0.03
	4	(0.47) ± 0.06	0.57 ± 0.03
	8	0.48 ± 0.06	0.57 ± 0.03
	14	0.45 ± 0.03	0.55 ± 0.03
	16	0.44 ± 0.03	(0.61) ± 0.06
	18	0.45 ± 0.03	–
	20	0.44 ± 0.03	–
	22	0.43 ± 0.03	–
16-SASL	–4	0.46 ± 0.03	0.55 ± 0.03
	0	0.44 ± 0.03	0.54 ± 0.05
12-SASL	34	–	0.54 ± 0.04
	29	–	0.52 ± 0.04

* Determined by subtracting the immobilized component to obtain a fluid component difference spectrum.

** Determined by subtracting the fluid (lipid-bilayer) component to obtain an immobilized component difference spectrum.

SASL taken from Ref. 1. It was not possible to determine a reliable value for the phospholipid probes, 16-PCSL and 16-PESL, because the underlying spin-spin broadened component yields too great an uncertainty in the baseline, even after subtraction, to give a faithful double integral. (For the same reason the integrations performed by Rousselet et al. [3] for spectra in the presence of fatty acid label micelles are also unreliable.) It is seen from Table I that there is no significant difference in the fractions of immobilized component between the steroid and the fatty acid probes. In view of the considerably different shapes and flexibilities of these two molecules, this result makes it unlikely that the major part of the immobilized spin label component arises from a specific binding of the spin labels to the protein. Taken together with the data on the phospholipid labels, the most likely interpretation seems to be that the immobilized component observed with all three different types of lipid molecule corresponds to the effects of lipid-protein interactions in the acetylcholine receptor-rich membranes. The result of which is to induce a selective immobilization in a substantial proportion of the membrane lipid population.

Because of the heterogeneity of the preparations it is difficult to give a more precise molecular description of the immobilized component. The acetylcholine receptor is the predominant protein species in the membrane and also the principal feature observed in negative contrast electron microscopy [10]. However, from the α-toxin binding activity it can be estimated that the receptor accounts for 20–25% of the total membrane protein. Thus it is possible that the greater part of the effects of lipid-protein interactions observed here may come from proteins other than the receptor. Nonetheless, it is of some interest to compare these results with recent structural data on the receptor protein, by making the assumption that the remainder of the protein has a similar structure. Chemical analysis yields the following lipid composition of the membrane: 0.77 μmol lipid phosphate/mg protein; 0.65 μmol cholesterol/mg protein. Thus an immobilized fraction $f = 0.45$, corresponds to approx. 86 phospholipid molecules and 74 cholesterols per 250000 dalton protein. The perimeter of negative-stain images of the receptor would occupy only approx. 54 molecules. However, neutron scattering [11], X-ray diffraction [12] and electron microscopy [13] data all suggest that the intramembranous section of the protein is much smaller than the 'rosette' structure which protrudes from the membrane and excludes negative stain. In a model such as that put forward by Wise et al. [11], approx. 190 lipid molecules could be accomodated under the area covered by this surface protrusion. Thus it appears that the internal structure of the receptor may be sufficiently complex to immobilize a rather large number of lipid molecules. Alternatively, the close-packing of protein molecules may cause more long-range lipid immobilization [1]. In either case it seems likely that the effect extends beyong a single shell of lipids surrounding the intramembranous perimeter, but without homogeneous preparations it is not possible to make firm statements. What does seem clear, however, is that the immobilization of the receptor protein seen by saturation transfer ESR [14], and presumably the densely-packed receptor regions at the synapse, arises from protein-protein rather than protein-lipid interactions [15–17].

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