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FREE FATTY ACIDS AND ESTERS CAN BE IMMOBILIZED BY RECEPTOR RICH MEMBRANES FROM TORPEDO MARMORATA BUT NOT PHOSPHOLIPID ACYL CHAINS

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SUMMARY : A long chain spin labeled fatty acid and the corresponding ester have been introduced into receptor rich membranes from Torpedo Marmorata. Superimposed to a mobile component, typical of the lipid phase, a strongly immobilized component is seen on the ESR spectra, both at low temperature (-4°C) and at room temperature. An estimation of the amount of immobilized signal as a function of the concentration of spin label in the membrane shows that a saturation is reached which corresponds to approximately twice the concentration of receptor protein. In the same membranes, a spin labeled phosphatidylcholine was introduced by the release of the phosphatidylcholine analog from purified phosphatidylcholine exchange protein, preloaded with this spin label. No immobilized component is seen in this latter case even at low temperatures. Therefore the immobilized component seen with the fatty acid cannot be considered as reporting on an immobilized boundary layer of phospholipids surrounding the proteins. We attribute the immobilized signal seen with fatty acids and esters to a particular interaction of amphiphilic molecules with the cholinergic receptor protein. Very likely this effect can be associated with the local anaesthetic effect detected previously with this fatty acid.

INTRODUCTION :

In 1975 we have shown that a spin labeled fatty acid can have pharmacological properties on Torpedo membrane fragments (1). Namely 8 doxyl palmitic acid behaves like a local anaesthetic as judged from electrophysiological experiments. At that time we were unable to show any evidence of the binding by electron spin resonance. Recently Marsh and Barrantes demonstrated that it is possible to see with spin labeled fatty acids incorporated into Torpedo membranes an immobilized component in addition to the fluid component which is found in aqueous bilayer dispersions of the extracted lipids (2). To observe the spectrum at low temperature (-4°C) helps to separate the 2 components whenever using 16 doxyl stearic acid; however using different spin labeled fatty

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acids the authors could show that very likely 2 components exist even at higher temperatures. Their interpretation of the results was that the immobilized component corresponded to a rigid phospholipid phase, which was assumed to be the total interstitial lipids occupying the area between the densely packed protein units in acetylcholine receptor rich membranes. In this article we will show that a spin labeled phospholipid, introduced in the membranes with the help of purified phosphatidylcholine protein does not give rise to an immobilized component. Furthermore the amount of immobilized signal obtained with a fatty ester (or acid) is saturable when the concentration of fatty ester is increased. The maximum number of immobilized spin labels corresponds to about twice the α toxin binding sites.

MATERIALS AND METHODS

Membranes enriched in cholinergic receptors were prepared from fresh electric organ from Torpedo marmorata, according to the method of A. Sobel et al. (3). The number of receptor sites was determined by the millipore filtration method (4); protein determination by the method of Lowry et al. (5) and phosphate titration by the method of Rouser et al. (6). The specific activity of our preparations was about 3 μ mole of ^{3}H α toxin binding sites/g of protein.

Spin labels : 16-doxyl stearic ester and acid were synthesized according to Hubbell and Mc Connell (7), with the nitroxide ring at the 16^{th} carbon atom. The corresponding spin labeled phosphatidylcholine was synthesized by reacting excess spin labeled anhydride with egg lyso lecithin (7).

Phosphatidylcholine exchange protein was purified from bovine liver as described in (8). It was stored at -10°C in 50% glycerol (vol/vol) at a concentration of 250 μg (8.6n mole of protein/ml).

Incorporation of the spin labels : The spin labeled fatty acid or ester was added to a glass tube from a concentrated ethanolic solution. The ethanol was dried under nitrogen and the membrane suspension added. This mixture was gently shaken for a few minutes and transfered to a 50 μl ESR flat quartz cell. The maximum amount of labels per membrane phospholipid was calculated to be < 1%. Double integration of the ESR signal showed that usely less label was present due to adsorption onto the glass tube.

In order to incorporate the spin labeled phospholipid, sonicated vesicles of this pure spin label were prepared (Ultrason Amemasse, 20' at 0°C under nitrogen), and freed of all fatty acid traces by incubation with a bovine serum albumin polymer (9). The purified exchange protein was mixed with the vesicles at 37°C. After one hour incubation, the phosphatidylcholine exchange protein was separated from the vesicles and glycerol on a column of Biogel A.0.5 M (Biorad Laboratories) equilibrated and eluted with Hepes sodium chloride buffer.

Phosphatidylcholine exchange protein was then mixed with the Torpedo microsacs at room temperature (2n mole cholinergic receptor plus 2 to 4 n mole of phosphatidylcholine exchange protein in 0.4 ml Torpedo ringer). The membranes were concentrated after 10 minutes incubation and transfered to an ESR quartz cell. Final spin labeled phosphatidylcholine concentration was 40 to 80 μMolar (obtained from double integration of spectra) for 40 μMolar cholinergic receptor. For experiments at -4°C, 10% sucrose was added to the Ringer solution. More details about the procedure of incubation with the phosphatidylcholine exchange protein will be published elsewhere (10).

ESR experiments : A Varian E 109 spectrometer connected to a Tektronix 4051 computer and provided with a field frequency lock was used to obtain the ESR spectra which were stored on tape. Spectra could be subtracted from each other and plotted on the XY recorder.

In order to estimate the amount of immobilized component present in the various samples, an arbitrary strongly immobilized signal, having an adequate extreme splitting (2T'//), was computer subtracted from the experimental spectra. This procedure was employed until evidence of a negative signal could be detected, particularly in the low field region. For more details on such quantitative estimations as first introduced by Jost et al. (11) see Davoust et al. (12). When very high concentrations of ester were used, it was found necessary to subtract another component corresponding to a single very broad line due to micelle formation or spin label segregation in the membranes which is likely to happen at low temperatures. As a result, in this latter case, a greater uncertainty was introduced in the determination of the ratio of immobilized versus free labels present in the membranes.

RESULTS

The ESR spectra of the stearic acid spin label and of the corresponding phosphatidylcholine spin label in acetylcholine rich membranes, at -4°C, are shown in figure 1A and B. The top spectrum is identical to that observed by

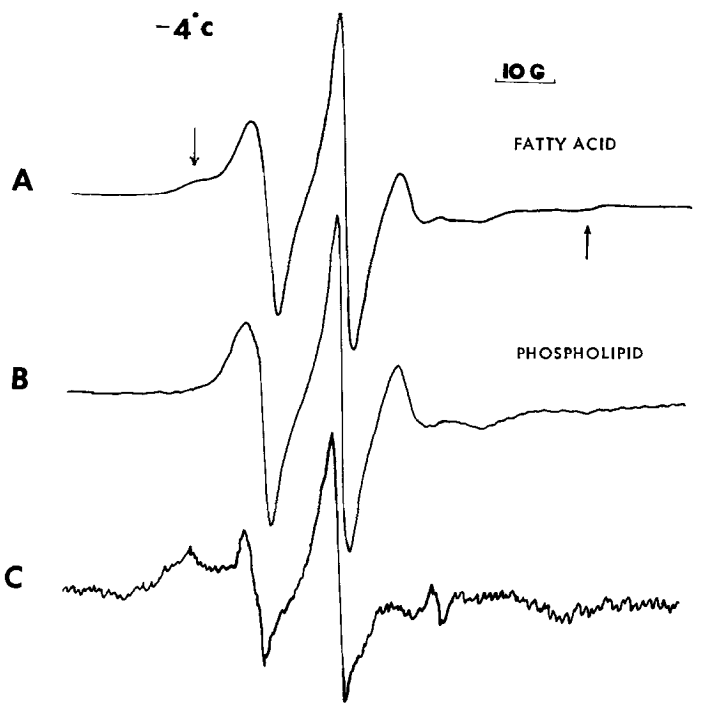

Figure 1 : ESR spectra of lipid spin labels in acetylcholine receptor rich membranes from Torpedo marmorata electric organ. The upper spectrum A is obtained with 16 doxylstearic acid at -4°C (the arrows indicate evidence of a strongly immobilized component). Middle spectrum B is obtained with the corresponding phospholipid. Spectrum C is the difference between A and B. Spin label concentration is equal to α toxine binding site concentration.40 µM.

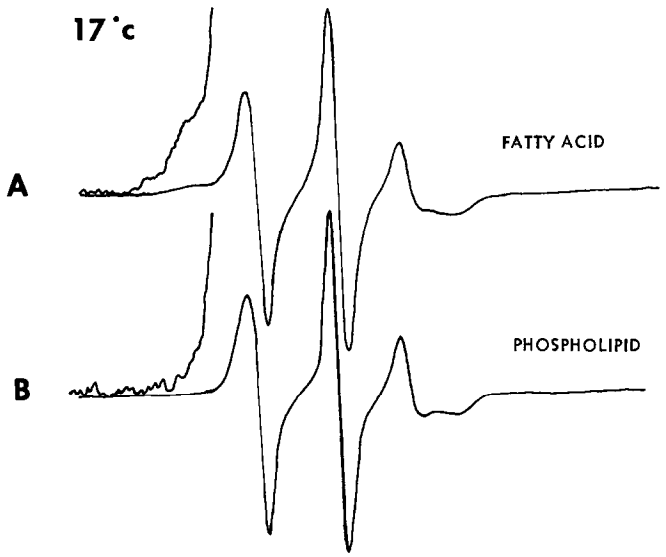

Figure 2 : Comparison of the ESR spectra of the fatty acid (A) and the phospholipid spin label (B) at 17°C in Torpedo membrane fragments. The low field region is enlarged to show the immobilized component present on the fatty acid spectrum (A).

Marsh and Barrantes (2). It can be obtained both with the fatty acid and the ester of 16 doxyl stearic acid. An immobilized component is clearly seen. It is indicated by an arrow on figure 1A. The spectrum B does not have the immobilized component. If it exists, it must correspond to a much smaller fraction (< 10%). Spectrum C is in fact the difference between A and B. It shows a characteristic immobilized signal superimposed with very narrow lines corresponding to the fatty acid in water. At the normal physiological temperature of Torpedo membranes (≃ 17°C), the immobilized component is also visible on the spectrum corresponding to the fatty acid but not on the spectrum obtained with the spin labeled phosphatidylcholine (figure 2). An estimation of the fraction of immobilized component in the various samples is summarized on Table I. A striking difference exists between the fatty acid and the phospholipid. No evidence of competitive effect of the local anaesthetic quotane with the spin labeled fatty acid or ester was found.

In the case of the ester, various concentrations of probes were tested in order to see if a saturation exists. Figure 3 shows the results. The uncertainty increases as the concentration increases due to micelle formation (see materials and methods). However it appears from figure 3 that it is not possible to assume a linear increase of the amount of immobilized component. The results suggest a saturation corresponding to about twice the concentration of receptor sites.

TABLE I

Spin label	T°C	% Immobilized spin label	
			+ quotane 1mM
16 Doxyl stearic acid	-4 +17	35 - 40 35 - 40	35 - 40
16 Doxyl stearic ester	0 +17	35 - 40 35 - 40	35 - 40
16 Doxyl phosphati - dylcholine	-4 +17	< 10 < 5	< 10

Spin label concentrations are equal to the concentration of $^{3}H\alpha$ toxin site present in the membrane suspension.

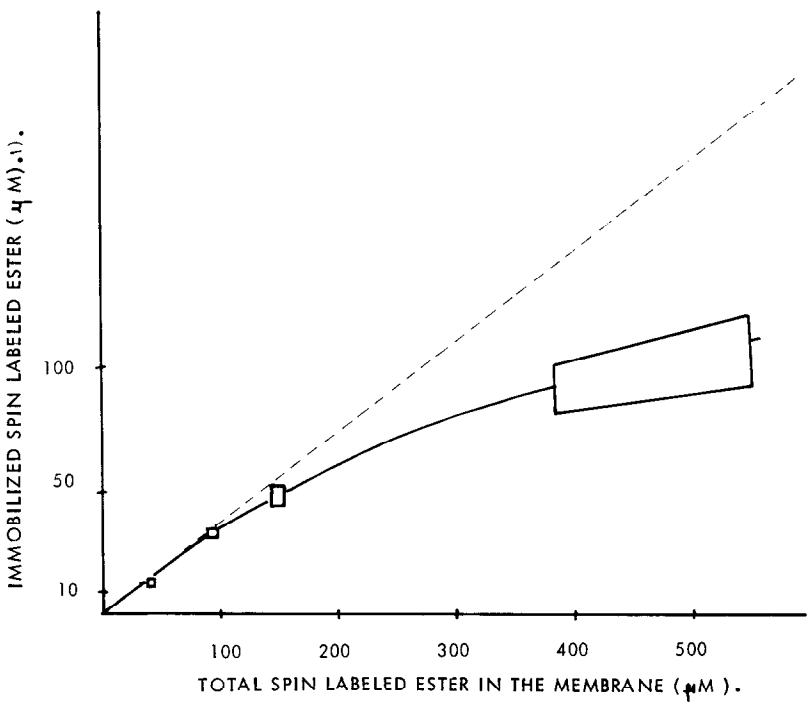

Figure 3 : Estimation of the immobilized spin label ester amount as a function of its total concentration in the membranes.

DISCUSSION

The results presented here show that in cholinergic receptor rich membranes from Torpedo marmorata, a fatty acid or ester can be strongly immobilized but that phospholipid chains are not.

In this study the spin labeled phosphatidylcholine is introduced with the help of purified phosphatidyl choline exchange protein. Such a procedure certainly does not perturbe the membrane structure. However it is likely that the labeled phospholipid is introduced only at the outer layer of the membrane (9). The fatty acid and the ester also penetrate the membranes on the outer layer but the flip-flop rate of a single chain is certainly much higher than that of phospholipids. Therefore one may question if the difference observed on the ESR spectra is due to differences in the distribution of spin labels. This assumption is very unlikely for the following reasons. Mc Namee and Mc Connell (13) have shown that in excitable microsacs of electric eel, which are comparable to the Torpedo membranes, the flip-flop rate of phosphatidylcholine is fast (a few minutes). Furthermore, we have recorded the spectrum given by the spin labeled phosphatidylcholine in the membranes after 12 hours incubation at room temperature, without seeing the appearance of an immobilized component.

The immobilized signal produced by fatty acid is better seen at low temperature (-4°C) and its quantitative estimation easier. Very likely the amount of immobilized component is identical at 17°C. However, the uncertainty is larger at this temperature, so that it cannot be ruled out that a small temperature dependance effect exists. We have shown, using a spin labeled fatty acid chain covalently bound to rhodopsin incorporated in egg lecithin, that low temperatures induce the appearance of an immobilized component. This immobilized component desappears at physiological temperature. This phenomenum is attributed to partial seggregation of the lipids at low temperature favouring protein-protein aggregation (14). The same explanation cannot be valid in the case of the Torpedo microsacs. Since the cholinergic receptor protein does not diffuse even at 30°C (15). As judged from electron micrographs protein are permanently in close contact.

We therefore conclude that fatty acids and esters have access to a special region of the cholinergic protein not accessible to a phosphatidylcholine. The immobilized component seen with a spin labeled fatty acid or ester would not be representative of a rigid phospholipid phase surrounding the proteins as suggested by Marsh and Barrantes (2). It would rather correspond to the binding of these amphiphilic molecules to the proteins. This conclusion is supported by the fact that fatty acids have a local anaesthetic activity which could imply the binding to an hydrophobic site of the proteins. It should be recalled that several toxins (crotoxin for example (16)) are site directed phospholipase A_2 enzymes. The toxicity seems to come from the release of fatty acids in the direct vicinity of the cholinergic receptor protein.

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