SATURATION TRANSFER ELECTRON PARAMAGNETIC RESONANCE ON MEMBRANE BOUND PROTEINS. II-ABSENCE OF ROTATIONAL DIFFUSION OF THE CHOLINERGIC RECEPTOR PROTEIN IN TORPEDO MARMORATA MEMBRANE FRAGMENTS

Annie Rousselet and Philippe F. DEVAUX

Institut de Biologie Physico-Chimique (ERA-CNRS n°690), 13, rue Pierre et Marie Curie, 75005 Paris, France

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SUMMARY : We have applied the technique of saturation transfer electron paramagnetic resonance to study the rotational diffusion of spin labeled membrane bound cholinergic receptors from Torpedo marmorata. Two different spin labels were used : a spin labeled maleimide derivative which binds covalently to proteins and a long chain spin labeled acylcholine which binds reversibly with a high affinity to the receptor protein. The maleimide spin label has a motion whose rotational correlation time is $\tau_2 > 10^{-3}$ sec. The long chain spin labeled acylcholine indicates slightly more motion ($\tau_2 \simeq 10^{-4}$ sec), but the nitroxide in this latter case is probably more loosely bound.

INTRODUCTION :

In a previous paper (1), we have shown that the saturation transfer spectroscopy can be employed to study the rotational motion of membrane bound rhodopsin. There has been up to now no direct measurement of the rotational motion of membrane bound cholinergic receptors. The lattice organization of the protein in Torpedo membrane fragments as detected by electron microscopy (2,3) and by X ray diffraction (4) suggests that the proteins are not free to diffuse in the plane of the membrane. In 1973 Bourgeois et al. (5) showed by high resolution autoradiography that the receptor protein in the subsynaptic membrane of electroplaque exhibits little tendency, if any, for lateral diffusion. Recent fluorescence photobleaching recovery experiments by Axelrod et al. (6) on membranes of developing muscle fibers show that the cholinergic receptors, in that system, can be clustered in patches and experience no lateral

Abbreviations : NEM : N ethyl-maleimide; MPTA : 4-(N-Maleimide)phenyl trimethylamonium; DTT Dithiol-threitol; TETRAM : O,O-diethyl S- (N,N-diethyl amino)ethyl phosphorothiolate; SDS : Sodium dodecyl Sulfate.

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diffusion. It may seem reasonable to anticipate in the case of Torpedo membrane fragments where the density of particles is high, that there is whether lateral nor rotational diffusion . However, it has been demonstrated that the lipid phase next to the cholinergic receptor experiences some fluidity (7). Therefore a direct measurement of the receptor rotational correlation time is a relevant experiment. Classical EPR spectroscopy does not allow to determine correlation times longer than 10^{-6} sec, thus we applied the saturation transfer electron paramagnetic resonance technique which allows the observation of slower motions (8).

MATERIALS AND METHODS :

Membranes enriched in cholinergic receptors were prepared from fresh electric organ of torpedo Marmorata as described by Sobel and Changeux (9). The number of receptor sites was determined by the millipore filtration method (10); protein determination by the method of Lowry (11); phosphate titration by the method of Rouser et al. (12). The specific activity of our preparations was about 3500 nmole of $^3H_\alpha$ toxin binding sites/g of protein. From the phosphate to protein ratio, there appears to be about 180 to 200 phospholipids per receptor site.

Spin labeling of the cholinergic receptor rich membranes. Spin label I (3-maleimido 2,2,5,5 tetra methyl 1-pyrrolidinyloxyl) was purchased from Syva (Palo-Alto). Infra-red spectroscopy showed no trace of the isomaleimide. Spin label II (8-doxyl palmitoylcholine) was synthetized according to Brisson et al. (13). When spin label I was used, the receptor rich membranes were preincubated for 15 hours at 6°C with NEM (1.5 excess toward receptor site concentration). After centrifugation at 100 000 g for 30 minutes, the membranes were treated one hour at 20°C with DTT (using two times the receptor concentration) and washed again with Torpedo Ringer. Following this treatment spin label I was added in the presence or in the absence of α-toxin from Naja-nigricollis (generous gift of Dr. P. Boquet). After 2 to 3 hours at 20°C, the unreacted spin labels were eliminated by centrifugation. The pellet was resuspended in the minimum volume and used for EPR experiments. The final concentration of receptor sites was of the order of 80 µM.

For SDS gel electrophoresis membranes were pretreated as previously describded with NEM and then with DTT, finally incubated with [^{3}H]-MPTA, a specific marker of the cholinergic receptor, (14) or with [^{14}C]-NEM.

When using spin label II membranes were preincubated with 10^{-4}M TETRAM a potent acetylcholinesterase inhibitor, for 30 minutes prior to the addition of the spin label. The receptor site concentration was always at least twice that of the spin label. Displacement of the specific probe from the cholinergic receptor sites was realized by the addition of 5 10^{-4}M acetylcholine.

EPR experiments. A varian E109 was used for the 1st harmonic in phase and the second harmonic out-of-phase. A 50µl quartz cell and a temperature control system were used. The detailed experimental conditions for saturation transfer spectroscopy are described in the previous paper (1) and in Thomas et al. (8). However, the much lower concentration of labeled protein available with the cholinergic receptor preparation (30µM instead of 300µM with rhodopsin) increases the time of accumulation. Two to three hours were required to obtain a correct signal to noise ratio for the second harmonic out-of-phase.

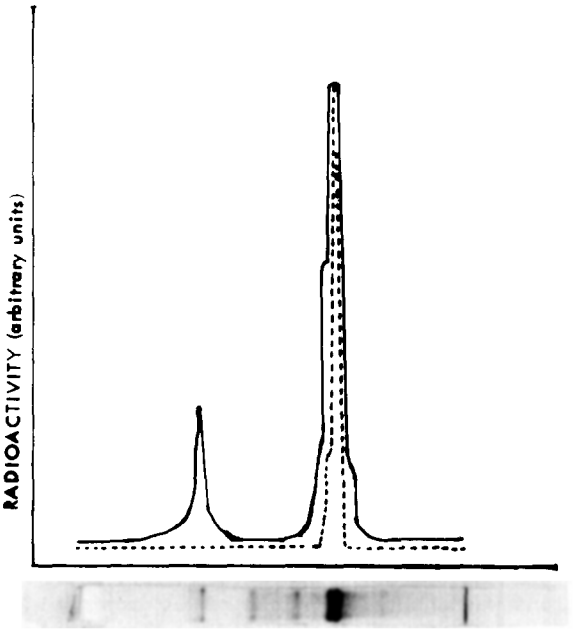

Figure 1 : Protein pattern of the receptor rich membranes of Torpedo Marmorata after SDS gel electrophoresis. The graph represents the radioactivity found in the different sections of the gel when the membranes are incubated with (^{3}H)MPTA a specific reagent of the receptor protein : ------ ; or when membranes are incubated with (^{14}C)NEM ——— (see text for detailed experimental conditions).

RESULTS :

Binding specificity of the maleimide derivatives labels.

In order to label preferentially the cholinergic receptor protein, we have developed a procedure (see Materials and Methods) which allows the labeling of the receptor disulfide bridge described by Karlin and Cowburn (14). Figure 1 shows a SDS gel electrophoresis pattern of Torpedo membranes. The band corresponding to the receptor protein (m.w. 40 000 - see reference 9) is given by the tritium peak of[^{3}H] MPTA (dotted line on figure 1). The distribution of [^{14}C] radioactivity (full line on figure 1) shows that about 50% of the [^{14}C] NEM binds to the receptor protein. However two other bands (m.w. 43 000 and 66 000) are also labeled.

Using this incubation procedure but replacing [^{14}C] NEM by spin label I, the same percentage of specific labeling is obtained. If membranes are spin labeled in the presence or in the absence of α-toxin a difference in the amount of binding appears. The double integration of the EPR spectra shows a 50% decrease of the number of spin labels bound when membranes are incubated in the presence of α-toxin. Assuming that α-toxin protects

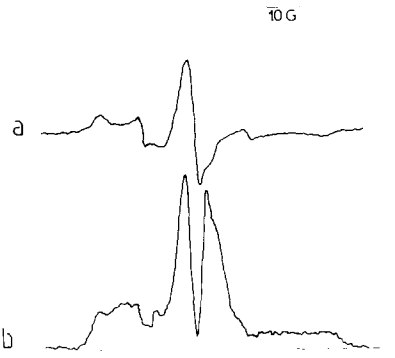

Figure 2 : EPR spectra of membranes labeled with spin labeled maleimide derivatives (spin label I). Top spectrum (a) is the first harmonic in-phase at 4°C (10 mw, 2 gauss, 100KHz), the maximum splitting is 68 Gauss. Bottom spectrum (b) : 2nd harmonic out-of-phase (32.2 mw, 5 Gauss, 50 KHz and three hours accumulation).

specifically the cholinergic receptor from the binding of the maleimide derivatives, it can be concluded that in the absence of α-toxin approximately half of the bound spin probes are on the receptor molecules.

EPR spectroscopy

A conventional 1st harmonic absorption spectrum of membrane fragments labeled with spin label I is shown on figure 2-a. Figure 2-b is the 2nd harmonic out-of-phase display. Spectra of figure 2 were recorded at 4°C. 1st and 2nd harmonic spectra of spin label I were also recorded at 20° and 35°C (not shown). Both types of spectra appear to be hardly influenced by the temperature in this range.

A small decrease of L"/H can be detected on the second harmonic spectrum at 35°C. But in the presence of sucrose, this difference disappears, indicating that it is probably due to vesicles rotation [¥].

Spin label II was shown to be a specific ligand of the cholinergic receptor (7,13). The very high specific activity of the present membrane preparation, and the low lipid to receptor protein ratio create good conditions to use this label. Figure 3-a_1 is the first harmonic absorption spectrum. It is remarkable that no signal of the probe in the aqueous phase can be seen, which indicates that a large proportion of the labels are bound to the protein. We calculated that more than 85% of spin

¥ The parameters L, L", H" and H correspond to the signal amplitude at the positions indicated by arrows (see Ref. 8).

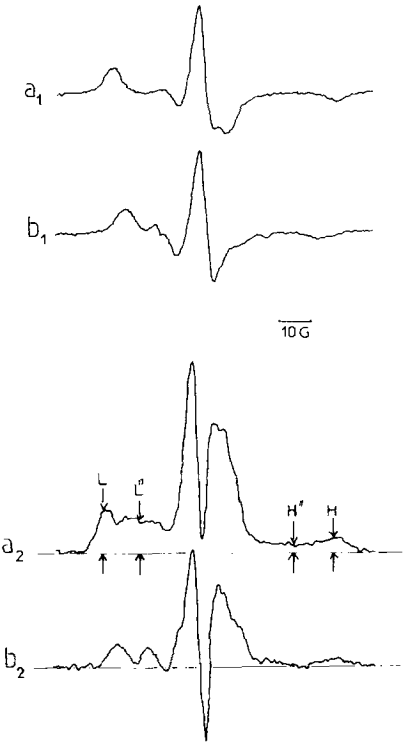

Figure 3 : EPR spectra of membranes labeled with a long chain acylcholine (spin label II). Temperature 4°C. The receptor site concentration is 80 µM and the concentration of spin labels 30µM. a_1 = 1st harmonic in phase; a_2 = 2nd harmonic out of phase; b_1 and b_2 : same conditions except that 5.10^{-4}M acetylcholine is added. The maximum splittings of the first harmonic spectra are respectively 68.5 G and 58.5 G.

label II was bound to the proteins, the rest being predominantly in the lipids. Figure 3-a_2 is the second harmonic out-of-phase spectrum. Spectra 3-b_1 and b_2 are the spectra recorded with the same sample but in the presence of 10^{-4}M acetylcholine.

DISCUSSION :

The signal obtained with spin label I corresponds to experimental conditions where, jugding from the assays with radioactive labels, a large fraction (≃ 50%) of the maleimide spin labels are attached to the receptor proteins. The signal obtained both in the first harmonic and the second harmonic mode reveals a complete immobilization. For instance H"/H = 1 and L"/L ≃ 1.1, which means according to reference (8) $\tau_2 >$

10^{-3} sec. Since there is no dependence on temperature of the shape of the spectra, it can be concluded that the motion corresponds strictly to $\tau_2 \cdot 10^{-3}$ sec.

Spin label II indicates by saturation transfer some motion of the probe. H"/H ≈ 0.5 and L"/L ≈ 0.8 which would mean $\tau_2 = 10^{-4}$ sec. However all the acylcholine spin labels are not bound to the proteins. From the value of the affinity coefficient we can calculate that about 15% of the spin labels are free to move in the lipids. The spectrum corresponding to such environment can be recorded by displacing all the probes from the receptor sites with 510^{-4}M acetylcholine. Finally by an adequate subtraction of EPR spectra, the pure signal of the protein bound spin labels can be obtained. Such procedure did not change significantly the values of H"/H and L"/L. Therefore the probe on the protein bound acylcholine experiences some motion. But it should be noted that the nitroxide in this latter case is separated from the binding site by 8 carbon atoms. It is likely to be less rigidly bound to the protein and to reflect less accurately its motion than spin label I. Indeed when 16-doxylstearoylcholine spin label was used (nitroxide 16 carbon atoms from the binding site) much more motion can be detected even with the 1st harmonic (7). We have attributed this motion to the vicinity of a fluid lipid phase near the cholinergic receptor protein.

The present interpretation of the saturation transfer spectra assumes that the reference spectra corresponding to isotropic motions can be utilized. This has been shown to be legitimate in the case of rhodopsin (1). The possible error in the measure of τ_2 by saturation transfer in the case of a cylindrical motion, depends on the average orientation of the principal axis of the spin label hyperfine tensor with respect to the protein rotational axis. If the two axis are parallel or almost parallel, the motion will be underestimated. In the present case, the maleimide probe is certainly bound at different protein sites, and the acylcholine has no reason to be oriented like the maleimide derivatives. Then it seems very unlikely that all the probes could be oriented on the proteins in the same unfavorable way (i.e. with the principle axis of the hyperfine tensor parallel to the axis of rotation). Therefore the absence of motion detected means truly no motion of the proteins.

To conclude the present study, it appears that in spite of the proximity of a fluid hydrophobic phase, the cholinergic receptor proteins are completely immobilized ($\tau_2 \cdot 10^{-3}$ sec). It should be noted that neither the physical state of the lipids in the membranes nor the size of the proteins can account for the differences in rotational diffusion of the

receptor as compared to the membrane bound rhodopsin. The average fluidity of the lipids of Torpedo membranes can be determined using various spin labeled fatty acids at different temperatures. It is found that there is no striking differences with the rod outer segment membranes (A. Rousselet, unpublished results). Since the molecular weight of the receptor complex is about six times that of rhodopsin molecules, a simple extrapolation from the rhodopsin results would lead to a correlation time τ_2 of the order of 10^{-4} sec for the rotation of free rosettes floating in a lipid bilayer. Hence strong protein-protein interactions must exist in the post synaptic membrane.

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