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EFFECTS OF TEMPERATURE, LIPID MODIFICATION AND pH ON THE MOBILITY OF THE MAJOR PROTEINS OF THE RECEPTOR-RICH MEMBRANES FROM *TORPEDO MARMORATA*

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The factors influencing the overall mobility of the major proteins of the acetylcholine receptor-rich membranes from *Torpedo marmorata* have been investigated by saturation transfer ESR spectroscopy and the lateral distribution of these proteins has been studied by electron microscopy. A spin-labelled derivative of maleimide, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (MSL), was used under various conditions of incubation, enabling us to attach it mainly to either an extrinsic protein of 43 kdaltons, or an intrinsic protein (40 kdaltons) bearing the α-toxin-binding site. (1) The direct reaction of MSL with the membrane fragments resulted in almost exclusive labelling of the 43 kdalton protein, an extrinsic protein located on the inner face of the receptor-rich membranes. (2) After the free SH groups were blocked with *N*-ethylmaleimide and the disulfide bridges opened with the reducing agent dithiothreitol, MSL reacted with both the 40 and 43 kdalton proteins (6.0 ± 0.6 MSL molecules per α-toxin-binding site). (3) After the latter labelling procedure membranes were exposed to pH 11, resulting in extraction of the 43 kdalton protein and leaving 2.2 ± 0.4 MSL molecules per α-toxin-binding site; sodium dodecyl sulfate polyacrylamide gel electrophoresis performed with *N*-[^{14}C]ethylmaleimide suggested that MSL was bound mainly to the 40 kdalton polypeptide chain of the acetylcholine receptor. The following conclusions were made with the native and alkaline-treated membranes: In the native membranes, saturation transfer ESR does not reveal any significant protein rotational diffusion (rotational correlation time $\tau_c > 1$ ms). Temperature variations and/or lipid modifications obtained by fusion of exogenous lipids and/or cholesterol exchange have little influence on the saturation transfer ESR spectra. Electron microscopy reveals that upon lipid addition, proteins remain in the form of clusters while areas depleted of proteins appear. On the other hand, alkaline treatment strikingly enhances the motion of the MSL-labelled proteins in the membrane ($100 \leqslant \tau_c \leqslant 120$ μs). Furthermore, the rotational diffusion of the MSL-labelled proteins (mainly the 40 kdalton protein) becomes sensitive to temperature, lipid composition and the lipid-to-protein ratio. Electron microscopy shows that alkaline extraction does not cause large reorganization of the acetylcholine receptor in the plane of the membrane. However, when phospholipids are added to pH 11 treated membranes, a dispersion of the receptor rosettes is observed. In contrast, cholesterol enrichment of the latter membranes induces clustering of the receptor and immobilization as judged by saturation transfer ESR. Upon reassociation of the pH 11 soluble proteins with the alkaline-treated membranes, the restriction of the acetylcholine receptor rotational mobility is also restored ($\tau_c \geqslant 1$ ms).

Abbreviations: MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl; SDS, sodium dodecyl sulfate; $C_{12}E_8$, dodecyl-octaoxyethylene glycol monoether.

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Introduction

ESR spectroscopy of nitroxide spin labels is a convenient method for investigating molecular motion of lipids and proteins in membranes. Used in a conventional manner, it yields information on rotational diffusion with correlation times shorter than 0.1 μs and is well adapted to the study of lipid chain mobility. The recently developed technique of saturation transfer ESR spectroscopy makes accessible significantly slower rotational correlation times (0.1 μs to 1 ms) and has been successfully applied to the measurement of the rotational diffusion of proteins in solution and in biological membranes [1].

Previous studies carried out using classical ESR spectroscopy [2–4] have shown that fluid lipids exist in purified receptor-rich membranes from *Torpedo marmorata*. These results suggest that the close packing of proteins, visible by electron microscopy, is primarily due to protein-protein interactions. However, Marsh and Barrantes [5] have suggested on the basis of spin-label data that the physical state of the lipids may be responsible for the organization of cholinergic receptor proteins in the postsynaptic membranes of *T. marmorata*.

We have carried out saturation transfer ESR experiments to study the rotational diffusion of the major proteins from receptor-rich membrane fragments. After labelling with (MSL), we showed in earlier studies that in native membrane no submillisecond movement takes place [6,7]. However, alkaline treatment which removed extrinsic proteins, mainly the 43 kdalton protein, induces rotational diffusion of the acetylcholine receptor [8]. This preliminary report was confirmed by Lo et al. [9]; the latter authors used the phosphorescence depolarization technique together with a labelled toxin. In the present article a more systematic study of the mobility of the major proteins of *T. marmorata* electric organ membrane fragments is reported. The influence of lipid addition and cholesterol depletion or enrichment in native of alkaline-treated membrane fragments is examined. Magnetic resonance studies are carried out in parallel with morphological examination by negative-staining electron microscopy.

Materials and Methods

Membrane preparation

Acetylcholine receptor-rich membrane fragments were purified from fresh *T. marmorata* electric organ as described by Sobel et al. [10] except that the sonication step was omitted. The specific activity of the fraction, determined as described in Ref. 10, was in general 2.5 ± 0.2 mol ^{3}H-labelled α-toxin-binding sites/g protein.

Spin labelling of acetylcholine receptor receptor-rich membranes

Procedure I. 1 μmol of the spin-labelled maleimide derivative MSL (Syva, Palo Alto), was added to the suspension of membrane fragments (1 ml at 2 mg protein/ml) and incubated for 12 h at 4°C under nitrogen. After elimination of the unreacted spin label by centrifugation, membranes were resuspended in the smallest volume of divalent cation-free buffer A (250 mM NaCl, 5 mM KCl, 10 mM Tris, pH 7.4) and used for standard ESR and saturation transfer ESR spectroscopy.

Procedure II. 1 μmol of unlabelled *N*-ethylmaleimide was added to the suspension of membrane fragment (1 ml at 2 mg protein/ml) and incubated overnight at 4°C under nitrogen. Unreacted *N*-ethylmaleimide was removed by centrifugation and the membranes were treated for 30 min at 20°C with 1 μmol dithiothreitol. After removal of dithiothreitol, 1 μmol MSL was added. The suspension was incubated overnight at 4°C, unreacted MSL was washed away and the suspension was equilibrated with buffer A.

Titration of MSL bound to the membrane was done by comparing the double integration of the spin-labelled membrane spectra with a reference. This double integration gives the total amount of spin label bound to the membrane fragments.

SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was done according to a modification of the procedure of Laemmli [11]. *N*-[^{14}C]Ethylmaleimide was used as the label instead of MSL.

Gels were stained with Coomassie brillant blue for protein detection. After protein staining, the gels were treated for fluorography and dried. Radioactiv-

ity was revealed on a Kodak X-Omat film after 48–150 h contact.

Alkaline extraction and reassociation of the 43 kdalton protein

Membranes labelled following procedure II were extracted with alkali using a method derived from that of Neubig et al. [12]. The suspension of membrane fragments in water at 4°C was rapidly adjusted to pH 11 with 0.1 M NaOH, incubated for 30 min at this pH and temperature and centrifuged for 45 min at 50 000 × *g*. The pellet was resuspended at pH 7.4 in buffer A and washed twice in this buffer. The pH 11 supernatant and the final membrane pellet were used for saturation transfer ESR spectroscopy. The extraction of protein was followed by SDS-polyacrylamide gel electrophoresis of the pH 11 treated membrane fragments. The pH 11 soluble proteins were reassociated with the alkaline-treated membranes following a modification of the method of Saitoh et al. [13]. Membranes spin labelled according to procedure II (8 mg protein) were exposed to pH 11 as previously described and subsequently mixed with pH 11 supernatant containing 8 mg protein and the *N*-ethylmaleimide-treated 43 kdalton protein. After overnight dialysis against 20 mM Tris-HCl buffer, pH 7.0, the suspension was mixed with egg phosphatidylcholine sonicated vesicles (1% of total membrane phospholipids) and 2% lipid-depleted bovine serum albumin (Sigma), and centrifuged for 1 h at 50 000 × *g*. The same procedure was followed with alkaline-treated membranes that had reacted with *N*-ethylmaleimide according to procedure II and reassociated with pH 11 supernatant containing the spin-labelled 43 kdalton protein. ESR spectroscopy was also performed with spin-labelled pH 11 supernatant reassociated with pure phosphatidylcholine vesicles and with aggregated 43 kdalton protein (see Results). Reassociation of spin-labelled 43 kdalton protein with sonicated phosphatidylcholine vesicles was done by mixing 2 mg of pH 11 soluble spin-labelled proteins (1 ml) with 1 ml of 1 mM sonicated phosphatidylcholine vesicles. The phospholipid-to-protein ratio was the same as in native membranes. After overnight dialysis, this sample was concentrated to 100 μl for saturation transfer ESR experiments.

The extent of reassociation of the pH 11 soluble proteins with the alkaline-treated membranes was followed with *N*-[^{14}C]ethylmaleimide-labelled 43 kdalton protein extracted at pH 11 from membranes labelled with *N*-[^{14}C]ethylmaleimide according to procedure I. Increasing amount of *N*-[^{14}C]ethylmaleimide-labelled pH 11 supernatant (10–400 μg protein) were added at pH 11 to a fixed amount (50 μg protein) of *N*-ethylmaleimide-labelled extracted membranes in a final volume of 1 ml. After overnight dialysis, this suspension was centrifuged and the pelleted membranes were resuspended in a constant volume. The radioactivity of the membrane pellet and supernatant was counted and the protein content was determined in each fraction. Protein composition of the pellets was followed by SDS-polyacrylamide gel electrophoresis.

Modification of the membrane lipid phase

Addition of phospholipids or depletion of cholesterol was done by fusing sonicated phospholipid vesicles with the various types of spin-labelled membrane fragments. Typically, 2 ml of the following suspensions of lipid vesicles were mixed with suspensions of membrane fragments containing 2 mg protein to achieve lipid transfer.

(a) 2 mM phosphatidylcholine/1.5 mM cholesterol for addition of phosphatidylcholine and cholesterol.

(b) 3 mM phosphatidylcholine for phosphatidylcholine addition and cholesterol depletion.

In these two instances, human plasma (previously heated at 56°C for 30 min was added to insure cholesterol exchange between vesicles and membranes [14].

(c) 2 mM phosphatidylcholine 0.5 mM phosphatidylserine/0.5 mM cholesterol for addition of phosphatidylcholine and phosphatidylserine. Phosphatidylserine was synthesized from phosphatidylcholine according to Ref. 15.

(d) 3 mM asolectin phospholipids for asolectin addition.

(e) 3 mM *T. marmorata* total phospholipids, extracted using the method of Popot et al. [16], for addition of total *Torpedo* lipids.

After incubation of the lipid vesicles with membranes for 12 h at 4°C under nitrogen, the membrane suspension was washed three times with buffer A to remove lipid vesicles adsorbed on the membrane surface. In some experiments, complexation of cholesterol into the membrane was achieved by

adding digitonin to a final concentration of 0.2%. Lysophospholipid introduction and phospholipid depletion were done by reacting membrane fragments with a phospholipase C (subunit B of crotoxin, a generous gift from Dr. C. Bon). The treatment performed as described by Marlas and Bon [17] resulted in the replacement of 10–50% of the membrane phospholipids by the corresponding fatty acids and lyso derivatives. Washing the phospholipase-treated membranes with a buffer containing 2% lipid-depleted bovine serum albumin extracted the phospholipid degradation products, yielding lipid-depleted spin-labelled membranes.

(f) Addition of detergent without solubilization: $C_{12}E_8$, a gift from Dr. M. Le Maire, was added to the spin-labelled membranes to a final concentration of 15% of the membrane phospholipids.

Acetylcholine receptor solubilization. A 250 μl aliquot of spin-labelled alkaline-extracted membranes (4 mg protein) was dissolved in 0.5 M octylglucoside (Sigma) in the presence of 1 mM sodium *p*-chloromercuribenzoate, and then centrifuged for 1 h at 100 000 × *g*. The clear supernatant was used for saturation transfer ESR experiments.

ESR determination of the relative amounts of exogenous phospholipids adsorbed and incorporated after fusion

Sonicated vesicles of pure spin-labelled phospholipids (16-doxylstearoylphosphatidylcholine) were mixed with *N*-ethylmaleimide-labelled *Torpedo* membrane fragments. The conventional ESR signal obtained with spin-labelled phosphatidylcholine-adsorbed vesicles is the same as that of pure spin-labelled phosphatidylcholine vesicles. This spectrum is very different from that obtained from diluted spin-labelled phosphatidylcholine in a biological membrane. The distinction between the two ESR signals allows subtraction of spectra and the measurement of the amount of incorporated and adsorbed phospholipids.

Total phosphorus in the lipid fraction was measured by the method of Rouser et al. [18]. Cholesterol was determined according to Ref. 19. Proteins were titrated using the method of Lowry et al. [20]. Results were expressed as mmol lipid incorporated/g membrane protein after correction for lipid adsorption.

ESR measurements

A Varian E 109 spectrometer was used in the absorption mode for the conventional ESR and saturation transfer ESR investigations. A 50 μl flat quartz cell was used together with a quartz Dewar. The temperature was controlled to within 1°C. For saturation-transfer ESR experiments, the incident microwave power on the sample was calibrated with Fremy's salts, in order to have an accurate value of the microwave field strength, $H_1^{eff} = 0.25 \pm 0.02$ G [21]. The modulation phase was adjusted before accumulation, using a very low microwave power (0.5 mW). The modulation phase was checked after the end of each recording. It was found to be stable even after 3 h accumulation. The minimum concentration of spin label used was 40 μM. The spectrometer was connected to a Tektronix 4051 computer which allows accumulation, subtraction and integration of spectra. A field frequency lock was systematically used for saturation transfer ESR registrations.

Electron microscopy

All samples were diluted prior to use in 13 mM ammonium formate to a final concentration of about 0.5 mg protein/ml. The negative-staining procedure was similar to that already described [22]. Thin carbon films supported on fenestrated plastic/carbon films were prepared according to Ref. 23. Uranyl formate was prepared as described in Ref. 24 and used as a 1% aqueous solution.

Observations were made with Philips electron microscopes EM 300 and EM 400 operating at 80 kV accelerating voltage and fitted with 50 μm objective apertures. Pictures were taken without preexposure of the speciment to the electron beam.

Results

Spin labelling of acetylcholine receptor-rich membranes

The membrane suspension was labelled with MSL following two distinct procedures (see Materials and Methods). In the first instance, membranes were mixed with MSL without any pretreatment; under this condition, MSL reacted with the free SH groups in the membranes. In the second instance, membranes were preincubated with unlabelled *N*-ethylmaleimide to block free SH groups, exposed to the

TABLE I

LABELLING OF *T. MARMORATA* RECEPTOR-RICH MEMBRANES BY MSL ON *N*-[^{14}C]ETHYLMALEIMIDE

	μmol/g protein		Number of molecules bound/α-toxin-binding site	
	MSL	*N*-[^{14}C]ethylmaleimide	MSL	*N*-[^{14}C]ethylmaleimide
Procedure I (MSL or *N*-[^{14}C]ethylmaleimide)				
Native membranes	43	28	21.5	14
After pH II treatment	6	5.5	1.2	1
Procedure II (*N*-ethylmaleimide – dithiothreitol – MSL or *N*-[^{14}C]ethylmaleimide)				
Native membranes	11.5	9.4	5.7	4.7
After pH 11 treatment	11.9	8.7	2.2	1.6

disulfide bond-breaking agent dithiothreitol and finally labelled with MSL; following this procedure MSL selectively labels the SH groups exposed by dithiothreitol treatment.

The amount of MSL bound to membrane fragments was determined by double integration of the conventional ESR spectra of the samples. The results are given in Table I. The amount of MSL bound using procedure I is more than 3-times larger than that with procedure II and corresponds to approx. 20 MSL molecules bound per α-toxin-binding site.

Alkaline treatment of acetylcholine receptor-rich membranes released the 43 kdalton protein into solution (Fig. 1). After this treatment, most of the MSL bound using procedure I was not recovered in the remaining membrane fraction (Table I) but in the supernatant. Accordingly, direct incubation of the membranes with MSL primarily labels the 43 kdalton protein. On the other hand, alkaline treatment of membranes labelled following procedure II resulted in the release of half of the label, the remaining half being present in about twice as many α-toxin-binding sites (Table I). In other words, after the use of procedure II, both the 43 kdalton protein and the receptor peptides are labelled in approximately equal amounts and a significant enrichment of the receptor-bound MSL takes place after alkaline treatment.

The selectively of labelling was checked by SDS-polyacrylamide gel electrophoresis of the membranes. Since MSL cannot be detected on gels after electrophoresis, parallel experiments were done with *N*-[^{14}C]ethylmaleimide. Table I shows that the efficiency of labelling with *N*-[^{14}C]ethylmaleimide

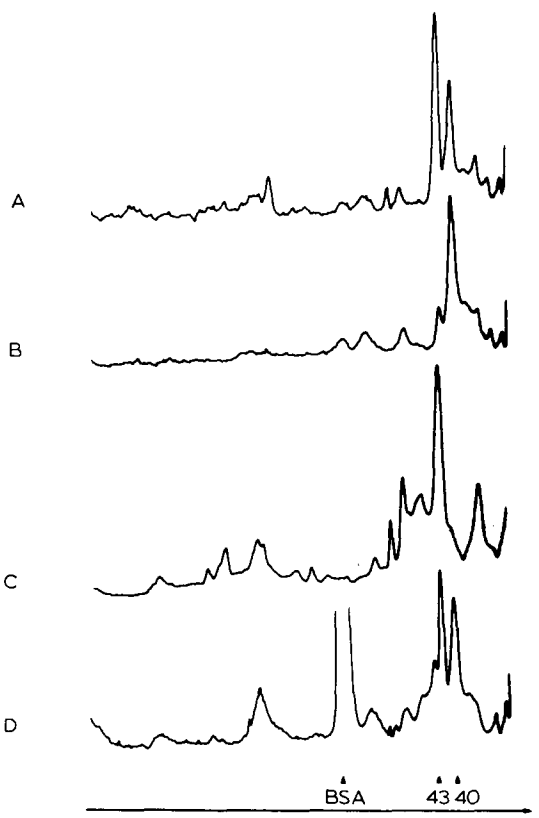

Fig. 1. Densitometric scans of SDS-polyacrylamide gel electrophoresis of: (A) native membranes, (B) alkaline-extracted membranes, (C) pH 11 supernatant, (D) extracted membranes reassociated with pH 11 soluble proteins. Note the presence, in D, of bovine serum albumin (BSA) which was added during the reassociation procedure (see Materials and Methods). Numbers refer to kdaltons.

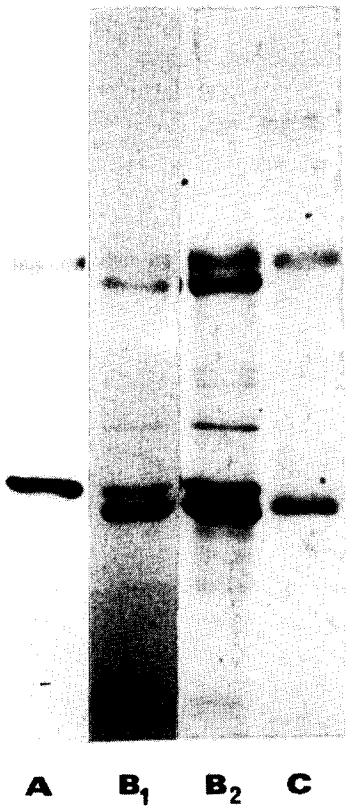

Fig. 2. Fluorographs of SDS-polyacrylamide gels of the various membrane samples. (A) Native membranes labelled according to procedure I (*N*-[^{14}C]ethylmaleimide only; 10 μg protein, 48 h contact). Specific activity 2 μM α-toxin-binding sites/g protein. (B_1 and B_2) Native membranes labelled using procedure II (*N*-[^{14}C]ethylmaleimide, dithiothreitol, *N*-[^{14}C]ethylmaleimide; 10 μg protein; B_1, 48 h contact; B_2, 150 h contact). 2 μM α-toxin-binding sites/g protein. (C) Alkaline-treated membranes labelled using procedure II (6 μg protein, 150 h contact). 5.5 μM α-toxin-binding sites/g protein.

was about the same as that found with MSL. The fluorographs of *N*-[^{14}C]ethylmaleimide-labelled membranes are presented in Fig. 2. Unambiguously, the 43 kdalton chain was the dominant polypeptide labelled by *N*-[^{14}C]ethylmaleimide in procedure I. On the other hand, following procedure II both the 40 and 43 kdalton polypeptides appeared radioactive on the gels as well as a few minor peptides such as the 95 kdalton one. After pH 11 treatment of these membranes, the 40 kdalton chain is the dominant polypeptide labelled by *N*-[^{14}C]ethylmaleimide. When bromoacetylcholine, and affinity reagent of the acetylcholine receptor site [25], was added to dithiothreitol-treated membranes prior to the addition of *N*-[^{14}C]ethylmaleimide, the extent of *N*-[^{14}C]ethylmaleimide labelling of the 40 kdalton polypeptide was reduced by more than 50%. Hence, in the pH 11 treated membranes most of the label is on the 40 kdalton protein and 50% of the 40 kdalton protein labelling is at the bromoacetylcholine-binding site.

Absence of protein mobility in native acetylcholine receptor-rich membranes: temperature effect

In agreement with the results of Rousselet and Devaux [6], the conventional ESR spectra of MSL bound to the receptor-rich membranes following procedures I and II are characteristic of a strongly immobilized nitroxide ($2T'_{//} = 67.5$ G) (data not shown). MSL is therefore expected to provide information about the rotational diffusion of the membrane proteins to which it is covalently bound.

Figs. 3A and 4A show the saturation transfer ESR spectra of native membranes labelled using procedures II and I, respectively. In these spectra, the height ratios, L″/L and H″/H, are the parameters which serve for the determination of the rotational correlation times of MSL-labelled proteins [21]. Table II gives the range of rotational correlation times estimated by comparing L″/L and H″/H experimental values with the reference values given by Thomas et al. [21] for standard globular proteins in aqueous solution. In this table, τ_L and τ_H are the values of the rotational correlation times determined by using, respectively, the low- and high-field part of the spectrum. The correlation times obtained with membranes labelled according to procedure I, where only the 43 kdalton protein was labelled, are typical for a slowly rotating protein (350–500 μs); those given for membranes labelled using procedure II, where both the 40 and 43 kdalton polypeptides were labelled, are characterized by the virtual absence of rotational diffusion in the time range explored (Higher limit of sensitivity 1 ms). The major components of *T. marmorata* receptor-rich membranes, the 40 and 43 kdalton proteins, are therefore strongly immobilized in the native membrane [6].

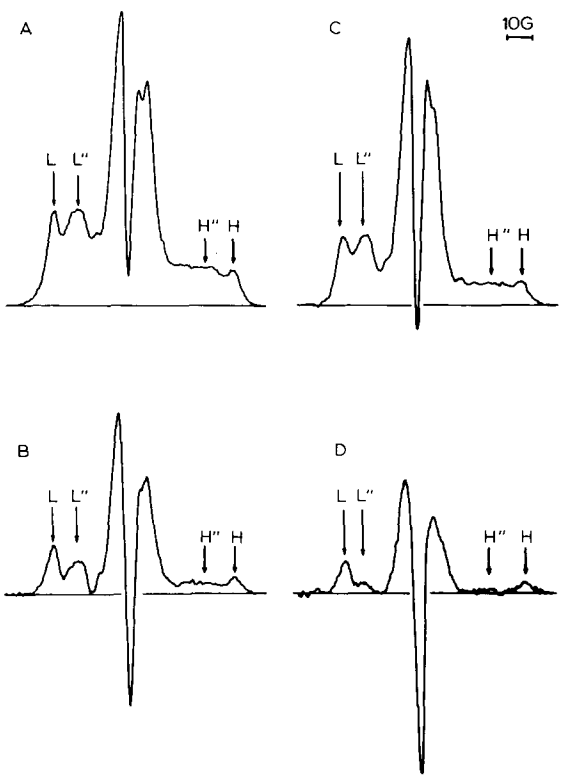

Fig. 3. Saturation transfer ESR spectra of receptor-rich membrane fragments labelled using procedure II (*N*-ethylmaleimide, dithiothreitol MSL). (A) Native membranes (40 mg protein/ml). (B) Alkaline-treated membrane fragments (20 mg protein/ml). (C) Alkaline-treated membrane fragments reassociated with unlabelled 43 kdalton protein (40 mg protein/ml). (D) Acetylcholine receptor soluble in octylglucoside after solubilization and high-speed centrifugation of alkaline-treated membrane fragments (16 mg protein/ml).

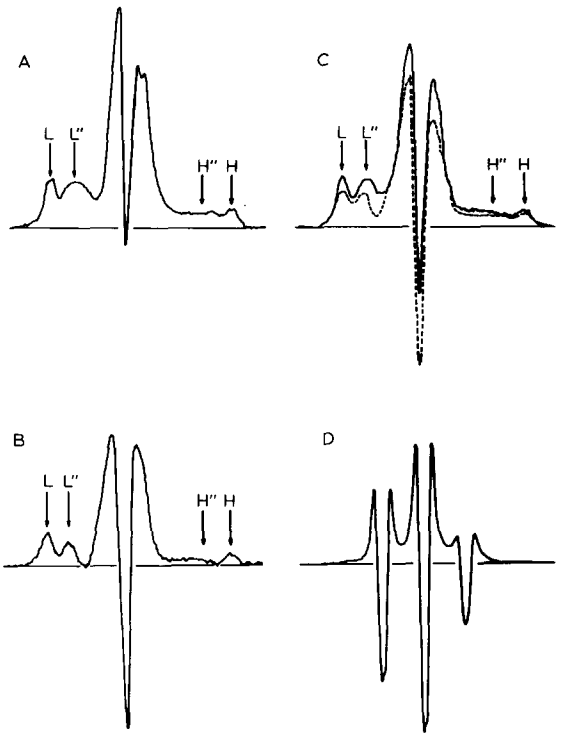

Fig. 4. Saturation transfer ESR spectra of receptor-rich membranes labelled using procedure I (MSL). (A) Native membrane fragment (20 mg protein/ml). (B) Spin-labelled 43 kdalton protein reassociated with sonicated phosphatidylcholine vesicles (0.5 mol phosphatidylcholine/mg protein; 20 mg protein/ml). (C) (———) Spin-labelled 43 kdalton protein reassociated with alkaline-treated, unlabelled membrane fragments (40 mg protein/ml). (- - - - -) Spin-labelled 43 kdalton protein aggregates obtained in the absence of extracted membranes at pH 7 with a high 43 kdalton protein concentration (25 mg/ml). (D) Soluble spin-labelled 43 kdalton protein at pH 11.

TABLE II

MSL ROTATIONAL CORRELATION TIMES IN VARIOUS MEMBRANE PREPARATIONS AT 17°C

	L''/L	Range of τ_L (μs)	H''/H	Range of τ_H (μs)
Procedure I (MSL)				
Native membranes	0.94 ± 0.04	200– 560	0.80 ± 0.04	300– 500
Procedure II (*N*-ethylmaleimide – dithiothreitol – MSL)				
Native membrane	1.02 ± 0.05	500–>1 000	0.96 ± 0.08	700–>1 000
After alkaline treatment	0.65 ± 0.05	32– 55	0.54 ± 0.02	100– 120
After solubilization by octylglucoside	0.37 ± 0.03	6– 10	0.3 ± 0.04	22– 32

The temperature dependence of the saturation transfer ESR spectra of membranes labelled by MSL according to procedure II was investigated. Fig. 5 shows that the effect of temperature could not be determined below 17°C, since the rotational motion of the labelled proteins was too slow. At 40°C a rapid decrease in τ_H took place (Fig. 5, ★) which, as shown by electron microscopy on a negatively stained preparation (Fig. 6), coincided with disruption of the receptor-rich membranes into small fragments. Interestingly, addition of sucrose (25%) to the heat-treated suspension caused an increase in correlation time τ_H from 200 to about 700 μs (Fig. 5, X). The same concentration of sucrose added to membrane fragments at 30°C had not effect. Thus, the exposure at 40°C did not cause a noticeable increase in intrinsic rotational motion of the membrane-bound protein but rather dispersed the receptor-rich membranes into pieces which, because of their smaller size, exhibited a faster motion than the native membrane fragments.

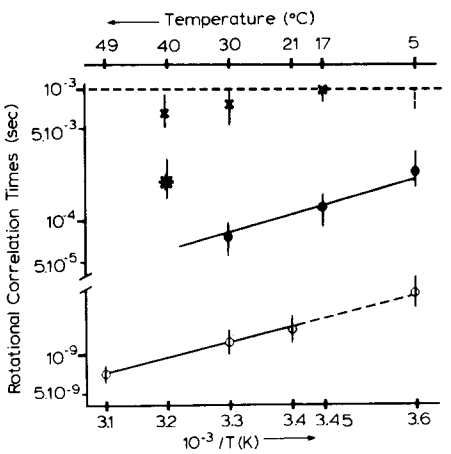

Fig. 5. Effect of temperature on the rotational correlation times of lipid and proteins in native and alkalin-treated membrane fragments. The rotational correlation time of acetylcholine receptor labelled using procedure II was plotted as a function of $1/T$ in native (X) and alkaline-treated (•) membrane fragments. (★) Rotational correlation time of acetylcholine receptor in native membranes measured at 40°C in the absence of 25% sucrose. The dashed line at 10^{-3} s show the upper sensitivity limit of saturation transfer ESR. Above this limit correlation times cannot be determined as in the case of native membranes below 17°C. The lower plot (o) represents the rotational correlation times of 16 doxylstearoylphosphatidylcholine, introduced into the *T. marmorata* membrane by the phosphatidylcholine-exchange protein [3], plotted vs. $1/T$.

Modification of the lipid phase

Phospholipids were added to native membranes by using the fusion procedure described in Materials and Methods. When pure spin-labelled phospholipids were used to measure the percentage of adsorbed phospholipid vesicles (Fig. 7), the broad line spectrum due to spin-spin interaction (Fig. 7A) was replaced by a composite spectrum (Fig. 7B) which revealed both adsorption and dilution of the exogenous labelled phospholipids into the *Torpedo* lipid bilayer. Linear combination of the spectra shown in Fig. 7A and B gave the spectrum shown in Fig. 7C; this three narrow line spectrum is typical of spin-labelled phosphatidylcholine diluted into a lipid bilayer. The percentage of the spectrum in Fig. 7A contributing to that in Fig. 7B provides a measure of the amount of adsorbed phospholipids. The quantities of phospholipids incorporated into the membrane after fusion (corrected or not corrected for adsorption) are listed in Table III. Thus, the addition of phospholipids to membranes results not only in adsorption of vesicles on the membrane surface, but also in the dilution of exogenous phospholipids into the *T. marmorata* membrane bilayer.

The incorporation of sonicate lipid vesicles into the membrane fragments was also followed by electron microscopy. To characterize the fused membranes on electron micrographs, a statistical population of membrane fragments in native and fused preparations was photographed and the number of acetylcholine receptor rosettes per square micron counted in each case. Fig. 8 shows the distribution of rosette densities in each type of preparation. It is clear that addition of phospholipids statistically lowers the particle density in the fused membrane fragments. When the phospholipids were added to the native membranes, the receptor rosettes remained in clusters, and a protein-free lipid area appeared (Fig. 9A). The same acetylcholine receptor clustering is also observed when native membranes are treated with 0.2% digitonin (Fig. 9B).

In Table IV are listed the values of H''/H and the ranges of rotational correlation times obtained after a variety of modifications of the lipid phase. The most obvious results is that the various lipid phase

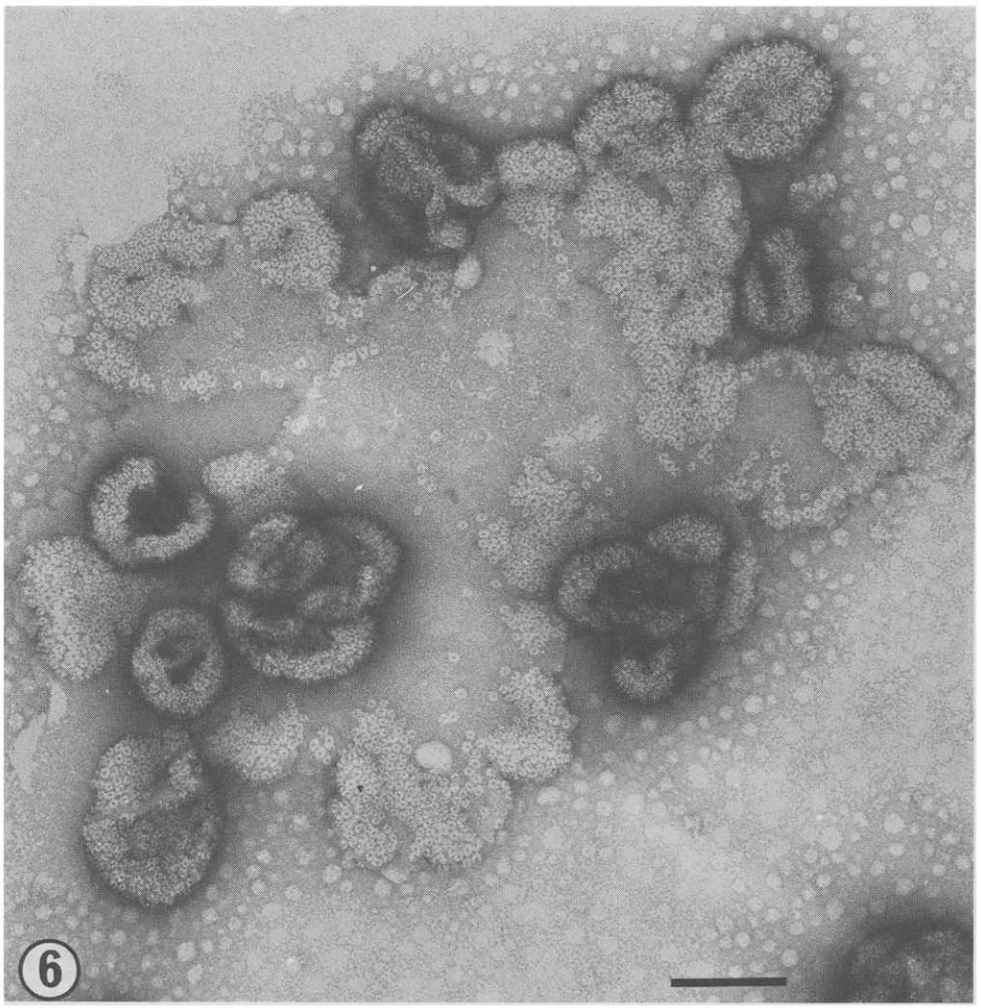

Fig. 6. Electron micrograph of negatively stained native *T. marmorata* receptor-rich membranes after 10 min incubation at 40°C. Note the persistence of the original fragment size determined by the disposition of the small vesicles (× 200 000). The bar represents 0.1 μm.

modifications investigated have little effect on the rotational diffusion of the proteins. Even after treatments as drastic as the addition of a detergent ($C_{12}E_8$) at a concentration equivalent to 15% of the membrane phospholipids or the present of 50% lyso derivatives, the MSL-labelled proteins remained immobile. The enrichment of cholesterol in the native membranes resulted in an increase in H″/H and L″/L, but the rotational correlation time of the acetylcholine receptor cannot be quantified in a reliable manner under these conditions (above the upper sensitivity limit of saturation transfer ESR spectroscopy).

Consequences of alkaline treatment on protein mobility of acetylcholine receptor-rich membranes

In agreement with the results of Neubig et al. [12] and others [26,27], alkaline treatment of the acetylcholine receptor-rich membranes results in almost complete elimination of the 43 kdalton protein and of a few higher molecular weight peptides (see Fig. 1). As a consequence, the acetylcholine receptor polypeptides become the dominant components of the membrane.

After this treatment no more than 5% lyso derivatives and fatty acids were detected in the membrane

TABLE III

INCORPORATION OF VARIOUS LIPIDS ACETYLCHOLINE RECEPTOR RECEPTOR-RICH MEMBRANES

Under the experimental conditions used, only one-half of the membranes presented the modified morphology shown in Fig. 9–11. Thus, the values of phospholipid incorporation given in this table should be multiplied by a factor of 2 in order to account for this nonrandom fusion.

	Total lipids present or added (uncorrected for adsorption) (mmol/g protein)		Lipids adsorbed (% of total lipids) added by ESR	Incorporated lipids after correction for adsorption (% variation vs. untreated membranes)	
	Phospholipid	Cholesterol		Phospholipid	Cholesterol
Control membranes	0.5 ± 0.05	0.45 ± 0.05			
+ phosphatidylcholine	0.7 ± 0.05	0.28 ± 0.05	47	+21 ± 2	−53 ± 2
+ phosphatidylcholine + cholesterol	0.7 ± 0.05	0.65 ± 0.05	60	+15 ± 2	+14 ± 2
+ phosphatidylcholine + phosphatidylserine + cholesterol	0.8 ± 0.05	0.5 ± 0.05	65	+21 ± 2	<1

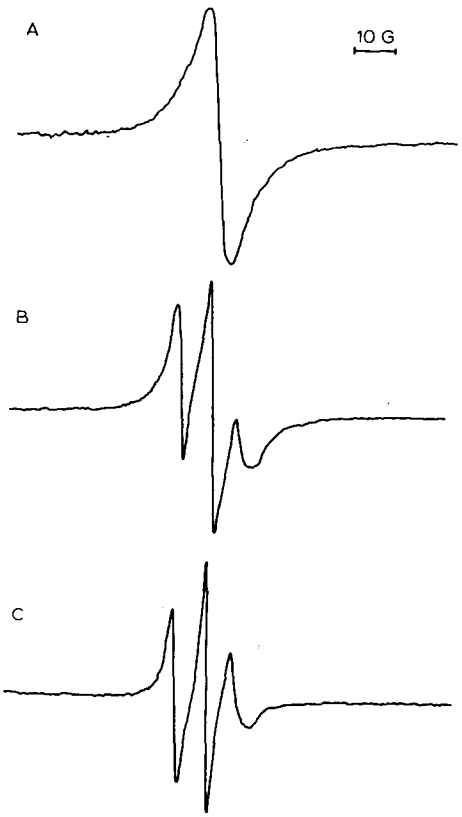

Fig. 7. Distinction between adsorbed and incorporated exogenous phospholipids. (A) Pure spin-labelled phosphatidylcholine vesicles. (B) Spin-labelled phosphotidylcholine after fusion with acetylcholine receptor-rich membranes. (C) Pure spectrum of membrane-incorporated spin-labelled phosphatidylcholine obtained by subtracting spectrum A from spectrum B.

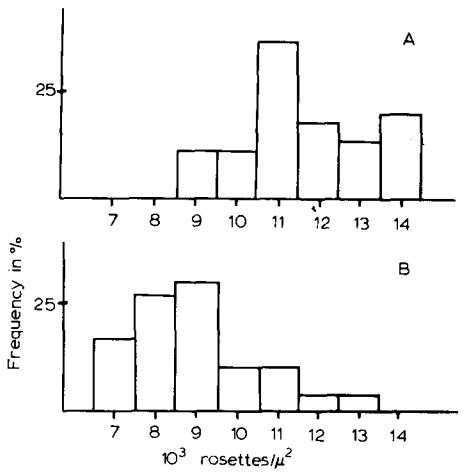

Fig. 8. Rosette density distribution in native (A) and fused (B) acetylcholine receptor-rich membrane obtained by a quantitative study of electron micrographs (see Results).

fragments. Fig. 3B and Table II show the ESR results obtained with pH 11 treated membranes labelled by MSL according to procedure II. The 'conventional ESR' spectrum was identical to spectra recorded with the original membranes. This implies that pH 11 treatment did not alter the immobilization of MSL by the proteins to which it is convalently bound. On the other hand, the saturation transfer ESR spectrum (Fig. 3B) reveals striking differences subsequent to alkaline treatment. Both L″/L and H″/H significantly decrease, indicating rapid rotational diffusion of the membrane-bound proteins. The rotational correlation time obtained (100–120 μs at 20°C) is still larger than that given by an octylglycoside-soluble protein extract of the same membranes (22–32 μs) (Fig. 3D).

Upon pH 11 treatment, the variation of the protein correlation time with temperature parallels that of a spin-labelled phosphatidylcholine, incorporated into the same membranes, suggesting that the mobility of the membrane-bound proteins has become dependent upon the lipid viscosity (Fig. 5). Electron microscopy of alkaline-treated membranes reveals no striking modifications, as pointed out already by Lo et al. [9]. The addition of phospholipids leads to dispersion of the receptor rosettes within the plane of the membrane (Fig. 11A–C). On the other hand, the addition of phospholipids together with a high ratio of cholesterol to phospholipid in the alkaline-treated membranes results in aggregation of the receptor rosettes into large clusters and in the formation of protein-free lipid areas (Fig. 10A and B). The latter phenomenon is accompanied by an increase in the rotational correlation time which reaches, a value close to that found in the native receptor-rich membranes (results not shown). Therefore, after extraction of the extrinsic proteins, acetylcholine receptor rational mobility is controlled by the lipids.

When the 43 kdalton protein is labelled according to procedure I, the pH 11 treated membranes show no ESR signal while the ESR spectrum of the supernatant is indicative of the motion of the spin-labelled 43 kdalton protein in solution. The conventional spectrum (not shown) is characteristic of a very mobile nitroxide and might result from a partial unfolding of

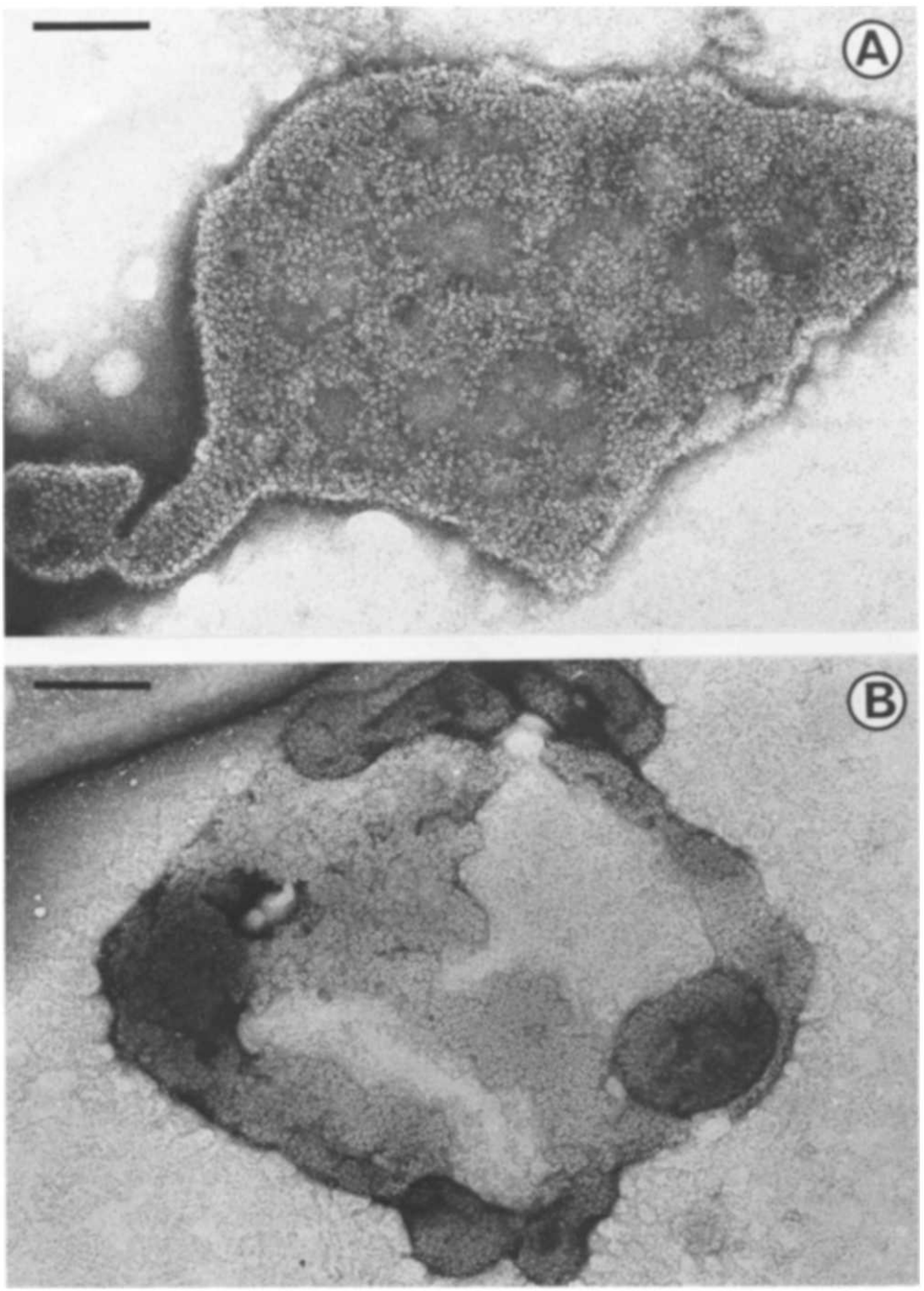

Fig. 9. Negatively stained acetylcholine receptor-rich membrane fragments after fusion with phosphatidylcholine alone and cholesterol depletion (A). Acetylcholine receptor-rich membrane fragments treated with 0.2% digitonin (B). × 160 000. Note the appearance of a protein-free area on the membrane surface.

TABLE IV

EFFECT OF LIPID PHASE MODIFICATIONS ON THE PROTEIN ROTATIONAL DIFFUSION IN NATIVE AND pH 11 EXTRACTED MEMBRANES LABELLED BY MSL (PROCEDURE II)

	H''/H	τ_H (μs) (17°C)
Native		
Control membranes	0.96 ± 0.08	700–>1 000
+ 20% phosphatidylcholine – 50% cholesterol + 20% asolectin + 15% *Torpedo* lipids + 20% phosphatidylcholine + 5% phosphatidylserine + 10% lysophospholipids + 50% lysophospholipids + 15% $C_{12}E_8$ + 0.2% digitonin	0.95 ± 0.1	500–>1 000
+15% phosphatidylcholine + 15% cholesterol –50% phospholipids	1.1 ± 0.1	>1 000
pH 11 extracted		
Control membranes	0.54 ± 0.02	100– 120
+ 20% phosphatidylcholine – 40% cholesterol	0.48 ± 0.02	70– 100
+ 15% $C_{12}E_8$	0.42 ± 0.02	55– 75
+ 20% phosphatidylcholine + 30% cholesterol	0.95 ± 0.05	800–>1 000

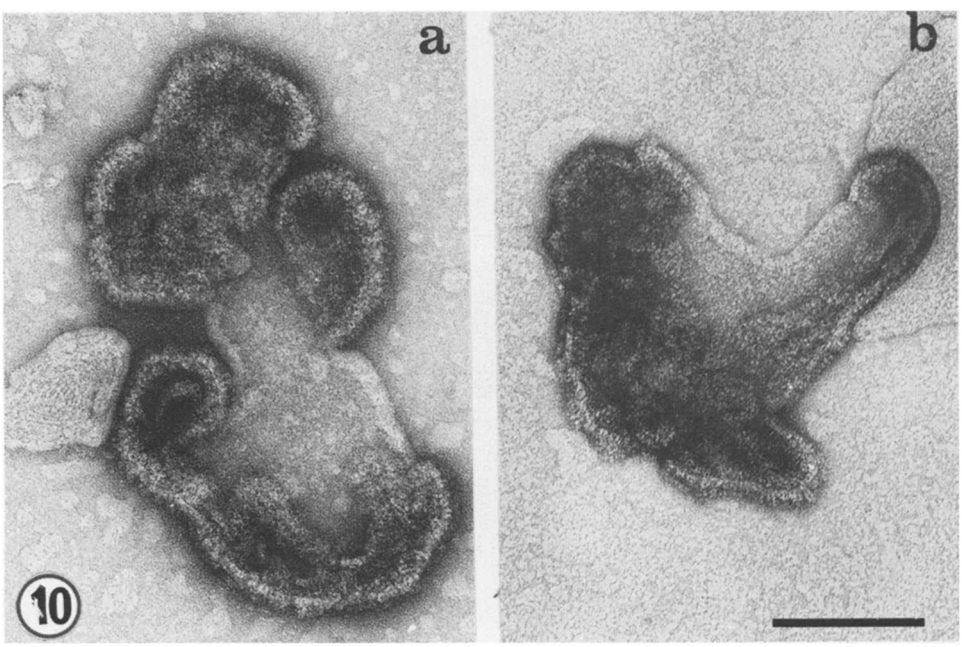

Fig. 10. Negatively stained alkaline-treated acetylcholine receptor-rich membranes after fusion with phosphatidylcholine and cholesterol. × 200 000. Note that the acetylcholine rosettes stay in clusters.

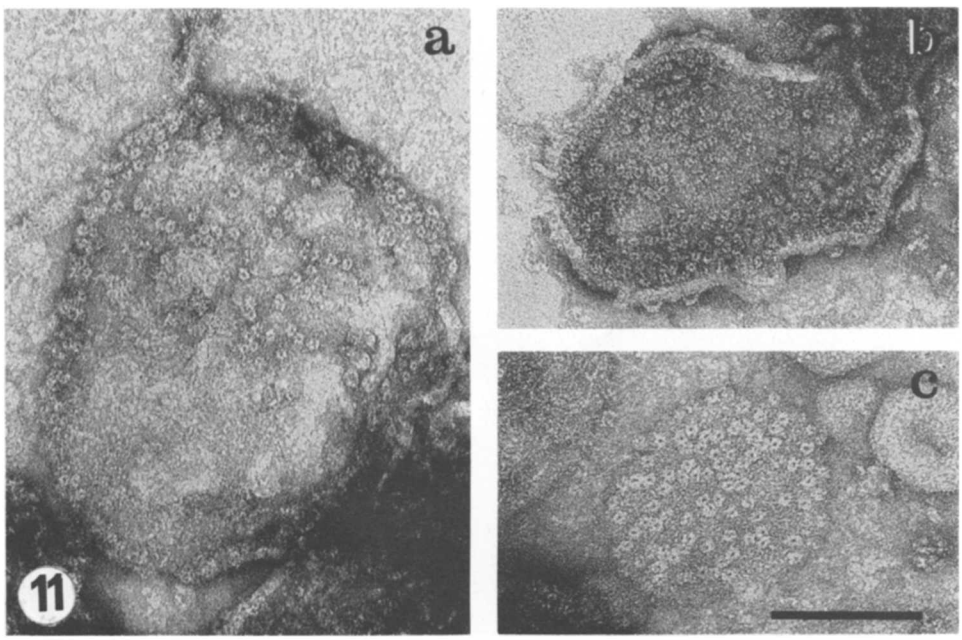

Fig. 11. Negatively stained alkaline-treated acetylcholine receptor-rich membranes after fusion with phosphatidylch ¹ine alone. × 200 000. The acetylcholine receptor rosettes are dispersed over the whole membrane surface.

the 43 kdalton protein at this high pH. Under these conditions, the rotational correlation time of the soluble 43 kdalton protein cannot be determined (see spectrum of Fig. 4D).

Consequences of the reassociation of the pH 11 soluble proteins with the alkaline-treated membranes

The reassociation of the pH 11 soluble proteins with the pH 11 treated membranes can be followed quantitatively with *N*-[^{14}C]ethylmaleimide-labelled 43 kdalton protein (see Materials and Methods). The variation of the ^{14}C-labelled 43 kdalton protein binding to alkaline-treated membranes as a function of the total concentration of pH 11 soluble proteins added was measured by radioactivity and monitored on SDS-polyacrylamide gels and fluorographs. The binding of *N*-[^{14}C]ethylmaleimide-labelled 43 kdalton protein to the alkaline-treated membranes tends to level off when high concentrations of 43 kdalton protein are added. This indicates that only a limited number of *N*-[^{14}C]ethylmaleimide-labelled 43 kdalton protein molecules may bind to the membranes. This limit corresponds to a mass ratio of 40 kdalton to 43 kdalton polypeptide close to 0.5. Partial reassociation of higher molecular weight proteins was also seen (Fig. 1).

Saturation transfer ESR spectra have been recorded with two 'reassociated' membrane preparations: in one case (Fig. 3C) the alkaline-treated membranes were labelled with MSL (following procedure II) and the 43 kdalton protein was not labelled. In the other case (Fig. 4C, full line) the 43 kdalton protein was spin labelled, but not the membranes. In both cases the spectra are characteristic of a decrease in motion (Table V) of protein-bound MSL, indicating that, as a consequence of reassociation, both the peptide(s) present in the alkaline-treated membranes and the added 43 kdalton protein became strongly immobilized. Alkaline-treated membranes were submitted to the reassociation procedure but in the absence of pH 11 soluble proteins. No immobilization of the acetylcholine receptor was seen. Thus, the reassociation procedure itself does not induce aggregation of the acetylcholine receptor.

In the absence of alkaline-treated membranes, the 43 kdalton protein forms large aggregates at pH 7 and

TABLE V

REASSOCIATION EXPERIMENTS WITH pH 11 SOLUBLE PROTEINS

	L″/L	Range of τ_L (μs) (17°C)	H″/H	Range of τ_H (μs) (17°C)
Labelled alkaline-treated membranes	0.65 ± 0.05	32– 55	0.54 ± 0.02	100– 120
Labelled alkaline-treated membranes + unlabelled 43 kdalton protein	1.02 ± 0.02	500–>1 000	0.93 ± 0.02	800–>1 000
Unlabelled alkaline-treated membranes + labelled 43 kdalton protein	0.94 ± 0.02	230– 360	0.88 ± 0.05	450–>1 000
43 kdalton protein aggregates	0.94 ± 0.02	230– 360	0.85 ± 0.02	350– 500
43 kdalton protein associated with phosphatidylcholine vesicles	0.67 ± 0.02	42– 50	0.48 ± 0.02	80– 100

at high protein concentration; under these conditions the saturation transfer ESR spectrum of the MSL-labelled 43 kdalton protein (Fig. 4C, dotted lines) gave similar correlation times, though the spectrum was different from that given by the 43 kdalton protein reassociated with the alkaline-treated membranes. In any case, this effect cannot account for the immobilization of MSL-labelled peptides of the alkaline-treated membranes after reassociation with the unlabelled 43 kdalton protein.

The reassociation of MSL-labelled 43 kdalton protein with sonicated phosphatidylcholine vesicles was also investigated using ESR spectroscopy. Under the conditions of reassociation (see Materials and Methods) the 43 kdalton protein was adsorbed to the lipid vesicles. To minimize the rotation of the vesicles, 25% sucrose was added to the suspension. The saturation transfer-ESR spectra recorded gave H″/H values compatible with rotational correlation times approx. 5–10-times shorter than those determined after reassociation of the 43 kdalton protein with the alkaline-treated membranes (Fig. 4B and Table V). 43 kdalton protein with the alkaline-treated membranes (Fig. 4B and Table V).

The most likely conclusion of the reassociation experiments following pH 11 treatment is that the immobilization of proteins in the native state of the receptor-rich membranes results from their interaction with the pH 11 soluble polypeptides and most probably the 43 kdalton protein; as a consequence of reassociation, the 43 kdalton protein itself becomes immobilized.

Discussion

The method of saturation transfer ESR gives reliable information about the rotational diffusion of macromolecules under a set of conditions defined quantitatively by Thomas et al. [21] for globular proteins in solution. It has been already shown that as a first approximation, the use of L″/L and H″/H leads to a reasonable estimation of rotational correlation times even for the anisotropic motions expected in biological membranes [28,29]. The central part of the saturation transfer ESR spectrum appears to be the most sensitive to motion but it is also sensitive to motion anisotropy and probe reorientation. As a consequence, large variations in the central part of the spectrum were not used to quantitate the changes in the rotational correlation time. The rotational correlation times τ_H and τ_L, estimated from the two ratios H″/H and L″/L, were nearly identical, although τ_L systematically was found to be slightly smaller than τ_H. This difference might result from the expected anisotropy of motion of the membrane-bound protein. It might also be due to the existence of a weakly immobilized MSL (5–10% of the conventional ESR signal integral) which interferes with the determination of L″/L.

Except after treatment of 40°C (which dispersed

the native membrane fragments into small pieces) the rotational correlation times measured were always insensitive to changes in the viscosity of the buffer caused by sucrose addition and therefore did not reflect the intrinsic rotation of the membrane fragments but rather that of their constituent proteins.

The membranes were labelled by a paramagnetic analog of *N*-ethylmaleimide which reacts preferentially with free SH groups on proteins. In the native membranes, most of the labelling took place at the level of the 43 kdalton protein. However, after blocking these sites with *N*-ethylmaleimide and treatment with the disulfide bond-breaking agent dithiothreitol, a significant fraction of the label was attached to the acetylcholine receptor site but also bound to a few other protein bands including the 43 kdalton protein. On the membranes labelled using this procedure and after elimination of the 43 and 95 kdalton proteins by alkaline treatment, a significant fraction of the label is expected to be bound and therefore to provide a measure of the rotation of the acetylcholine receptor. Some of the ESR results presented previously [6,8] and in this paper have been suggested by Barrantes et al. [30] and confirmed by Lo et al. [9] with the same material * using the method of phosphorescence depolarization. These results lead to the conclusion that immobilization of the intrinsic membranes proteins in *T. marmorata* subsynaptic membrane fragments comes from their interaction with the extrinsic proteins, particularly the 43 kdalton protein. Morphological observations presented by Cartaud et al. [35] and the effects of selective proteolysis of the same membranes [36] reveal, in addition, that this interaction takes place on the inner cytoplasmic face of the membrane. On the other hand, Saitoh et al. [13] have demonstrated that reassociation of alkaline-treated membranes with pH 11 soluble proteins protects the acetylcholine receptor from heat denaturation. At this level, cross-linking of the membrane proteins and in particular of the acetylcholine receptor by the 43 kdalton polypeptide appears sufficient to abolish the rotational diffusion of the two partners. In native membranes, a slight difference between the extrinsic 43 kdalton protein and instrinsic protein correlation times was noticed. This difference might be due to a change in motion anisotropy which is plausible with an extrinsic protein like the 43 K protein rather than resulting from an intrinsic difference in rotational diffusion.

The importance of protein-protein interactions rather than protein-lipid interactions in the maintenance of the stability of the subsynaptic membrane is also illustrated by the low sensitivity of the protein rotational diffusion to lipid phase modifications in the native membranes. Addition of phospholipid up to 40% (Fig. 8A) does not cause a 'dispersion' of the receptor rosettes. Thus, in the native membrane, the high protein-to-lipid ratio is not responsible for the close packing of the receptor molecules as it occurs in reconstituted membranes with rhodopsin [37,38] or bacteriorhodopsin [39,40]. On the other hand, after removal of the 43 kdalton protein, the rotational motion of the remaining membrane proteins, the acetylcholine receptor in particular, becomes sensitive to the state of the lipid phase. Still apparently closely packed in the alkaline-treated membranes, the receptor rosettes were partially dispersed by the addition of exogenous phospholipids to these membranes. Then addition of phospholipids to and cholesterol depletion from pH 11 treated membranes allow both the rotational and the lateral diffusion of the acetylcholine receptor rosettes. Furthermore, addition of cholesterol to the alkaline-treated membranes causes aggregation of the rosettes associated with the loss of rotational diffusion. Thus, cholesterol plays a particular role in the organization of receptor-rich membranes. That cholesterol might interact with the receptor protein was shown already by different groups using various approaches. For exaple, Popot et al. [16] have shown that the purified receptor protein reassociates preferentially with cholesterol-containing monolayers. More recently, using planar bilayers Schindler and Quast [41] demonstrated that the cooperativity of purified acetylcholine receptor reconstituted in such membranes was enhanced by

* We have used membranes prepared without EDTA. Under these conditions mild proteolysis of the β, γ and δ chains occurred, giving a simplified pattern in SDS-polyacrylamide gel electrophoresis [31,32]. As shown by Lindstrøm et al. [33], low concentrations of papain applied to acetylcholine receptor-rich membranes have no effect on receptor function and morphology. Only strong trypsin treatment [34] has an effect on the size of the membrane vesicles. Thus, the mild proteolysis present in our preparation does not interfere with the results presented.

cholesterol. Finally, Marsh and Barrantes [5] have shown that a spin-labelled cholesterol can be immobilized by the receptor protein, which suggests a specific association.

In conclusion this study offers two plausible, but nonexclusive, mechanisms for the aggregation and stabilization of the receptor protein in the subsynaptic membrane during synapse formation: (i) self-aggregation facilitated by cholesterol and (ii) cross-linking by an extrinsic protein, most likely the 43 kdalton protein.

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