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The rotational diffusion of the acetylcholine receptor in *Torpeda marmorata* membrane fragments studied with a spin-labelled α-toxin: importance of the 43 000 protein(s)

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The rotational diffusion of the acetylcholine (ACh) receptor in subsynaptic membrane fragments from *Torpedo marmorata* electric organ was investigated with a spin-labelled α-bungarotoxin. A toxin with two spin labels was first synthesized; the conventional electron spin resonance spectrum (e.s.r.) of this toxin bound to the receptor indicated: (1) a complete immobilization of the probes; and (2) a strong spin-spin interaction that was not, or barely, seen in solution. The modification of the degree of spin-spin interaction is taken as an indication of a toxin conformational change accompanying its binding to the ACh-receptor. To avoid spin-spin interaction a single-labelled toxin was made and used to follow the rotational diffusion of the receptor by saturation transfer e.s.r. (ST-e.s.r.). With native membranes a high immobilization of the ACh-receptor was noticed. Reduction of the membranes by dithiothreitol had little effect on this motion. Only extraction of the 43 000 protein(s) by pH 11 treatment was able to enhance the rotational diffusion of the ACh-receptor protein (rotational correlation time by ST-e.s.r. in the 0.5 – 1 x 10^{-4} s range) and to allow its lateral diffusion in the plane of the membrane fragments (observed by electron microscopy after freeze-etching or negative staining).

Key words: acetylcholine receptor/dithiothreitol/electron microscopy/43 000 protein(s)/saturation transfer electron spin resonance

Introduction

In the sub-synaptic membrane of the neuromuscular junction (for review, see Fertuck and Salpeter, 1976) and of the electromotor synapse (for review, see Bourgeois *et al.*, 1978), acetylcholine (ACh)-receptor molecules are densely packed, while outside the synapses their density is 100 – 1000 times lower. Denervation experiments (Bourgeois *et al.*, 1973, 1978; Frank *et al.*, 1975) show that these dense accumulations of receptor persist for days (or even weeks) without signs of lateral diffusion. Absence of lateral diffusion of the ACh-receptor in the patches which form spontaneously on cultured rat myotubes was also observed by Axelrod *et al.* (1976). Investigations on the rotational diffusion of the ACh-receptor performed by Rousselet and Devaux (1977), *in vitro*, on purified sub-synaptic membrane fragments prepared from *Torpedo marmorata* electric organ using saturation transfer electron spin resonance (ST-e.s.r.) and a spin-labelled maleimide (MSL), did not reveal any sub-millisecond rotation of the labelled proteins that are thus strongly immobilized in these fragments.

Several mechanisms might plausibly account for this immobilization: (1) an interaction of the ACh-receptor with the basal lamina on its outside surface (Burden *et al.*, 1979); (2) a crosslinking of ACh-receptor molecules by cytoplasmic peripheral proteins which might be related to the cytoskeleton (see for the erythrocyte membrane: Branton *et al.*, 1981); or (3) an intervention of immobilized protein-bound lipids (Marsh and Barrantes, 1978). The first mechanism cannot be involved with isolated membranes that are no longer covered with a basal lamina. To test the third mechanism, spin-labelled long chain acylcholines were synthesized (Bienvenüe *et al.*, 1977): they disclosed that the lipid environment of the ACh-receptor is fluid. Furthermore, incorporation of spin-labelled phospholipids *via* fusion or *via* an exchange protein did not reveal any immobilized lipids in *Torpedo* membrane fragments (Rousselet *et al.*, 1979a; McNamee *et al.*, 1982). Axelrod *et al.* (1978a, 1978b) also demonstrated with chick embryo cultured myotubes that lipid modifications do not cause lateral diffusion of the patched receptors labelled with fluorescent α-toxins. In contrast, Marsh and collaborators have reported immobilized phospholipids in *Torpedo* membranes (Marsh and Barrantes, 1978; Marsh *et al.*, 1981).

ACh-receptor rich membranes from *Torpedo* give, after denaturing polyacrylamide gel electrophoresis in one-dimension, a pattern of protein bands characteristic of the four chains of the ACh-receptor. In addition, a band of apparent mol. wt. 43 000 (Sobel *et al.*, 1977, 1978), distinct from actin (Sobel *et al.*, 1978; Strader *et al.*, 1980), is present. On two-dimensional gels this protein (named ν by Karlin *et al.*, 1979) yields one major component that focuses at alkaline pH and others that focus at more acidic pH (Saitoh and Changeux, 1980). The first (named ν_1 by Gysin *et al.*, 1981) at variance with the others (ν_2 and ν_3) is not present in the cytoplasm (Gysin *et al.*, 1981) and appears strictly membrane-bound. Brief exposure of the membrane fragments to pH 11 releases this protein without interfering significantly with the main functional properties of the still membrane-bound ACh-receptor (Neubig *et al.*, 1979).

Evidence that the 43 000 (ν_1) protein plays a "structural" role in the sub-synaptic membrane was first suggested by the observation that its removal enhances the thermal inactivation of the membrane-bound ACh-receptor (Saitoh *et al.*, 1979) and its digestion by proteolytic enzymes (Klymkowsky *et al.*, 1980). To test the second mechanism considered above, of a cross-linking of the receptor by peripheral proteins, the rotational diffusion of the main proteins present in the ACh-receptor rich membrane fragments was measured by ST-e.s.r. (Rousselet *et al.*, 1979b, 1981). Membranes were first reduced by dithiothreitol (DTT) and then reacted with MSL: most of the MSL bound to the α chain of the ACh-receptor and to the 43 000 protein(s). Alkaline treatment resulted in an increase of the rotational diffusion of the membrane-bound ACh-receptor. By varying the lipid composition by fusion, it was also shown that phospholipids do not play a major role in the immobilization of the ACh-receptor, underlying the impor-

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tance of protein-protein interactions for the maintenance of the post-synaptic organization (Rousselet *et al.*, 1981). An increase in rotational diffusion of the ACh-receptor after alkaline extraction was also demonstrated by phosphorescence depolarization (Lo *et al.*, 1980). Finally, electron microscopy showed a reorganization of the receptor rosettes in the plane of the membrane after alkaline extraction (Rousselet *et al.*, 1979b; Barrantes *et al.*, 1980; Cartaud *et al.*, 1981).

Selective proteolysis (Wennogle and Changeux, 1980) and iodination (Saint-John *et al.*, 1982) experiments show that these 43 000 protein(s) bind to the inner, cytoplasmic, face of the ACh-receptor rich membranes and might be component(s) of the cytoplasmic condensations observed after tannic acid coloration (Cartaud *et al.*, 1981; Sealock, 1982).

The structural role of the 43 000 protein(s) was recently challenged by Bartholdi *et al.* (1981), who, working with membrane fragments labelled by a phosphorescent α-toxin, detected apparent changes of motion of their probe only at very high temperature with pH 11 treated membranes, or after reduction of intact membranes with DTT. We have thus reinvestigated this question by both electron microscopy and ST-e.s.r. using a highly selective probe of ACh-receptor rotational diffusion: a spin labelled α-bungarotoxin which gives strongly immobilized signals when it binds to the ACh-receptor. The data confirm that the immobilization of the ACh-receptor results from protein-protein interactions involving the internal 43 000 protein(s).

Results

Spin labelling of α-bungarotoxin and interaction of the labelled toxin with the membrane-bound ACh-receptor

When α-bungarotoxin is exposed to stoichiometric amounts of DTT, the cysteine 30–cysteine 34 "extra"-disulphide bridge exclusively opens (Botes, 1974). The resulting free-SH groups can then be alkylated by a maleimide spin label (^{2}H,MSL) yielding (Method 1): 2 ± 0.1 spin labels per α-toxin molecule. H.p.l.c. of the labelled toxin gives a single major peak that contains 90% of the protein applied on the column.

In Figure 1, the spectra of the double-labelled toxin are compared, free in aqueous solution (a – full line) and bound to the ACh-receptor rich membrane fragments (b and c). The spectrum of the bound toxin revealed a strong immobilization of the probes. This spectrum shows that the binding of α-bungarotoxin was tight at the level of these cysteines. In addition, spectrum 1b showed that the two ^{2}H,MSL labels of the toxin, which were magnetically independent in the water soluble state, now strongly interacted. Two alternative explanations may account for this interaction. Since, at saturation, two molecules of toxin bind per molecule of ACh-receptor, the interaction might take place between labels from different toxin molecules. Alternatively, the two labels from the same toxin molecule could interact with each other. To distinguish between these possibilities, the membrane fragments were first treated with unlabelled α-toxin and then exposed to the paramagnetic toxin. As a result, at most 30% of the sites were labelled by ^{2}H,MSL-toxin. Under these conditions, the spin-spin interaction remained unchanged indicating that this interaction occurred between the labels attached to the same toxin molecule, i.e., it was an intramolecular process. Finally, after saturation of the membrane fragments with unlabelled toxin, no immobilized component was observed upon addition of the labelled toxin (Figure 1e), which could then be eliminated by centrifugation. Addition of 1 M carbamylcholine to the ^{2}H,MSL toxin-membrane complex yielded, after 45 min, the same free toxin spectrum as in Figure 1c indicating that the totality of the labelled toxin was bound to

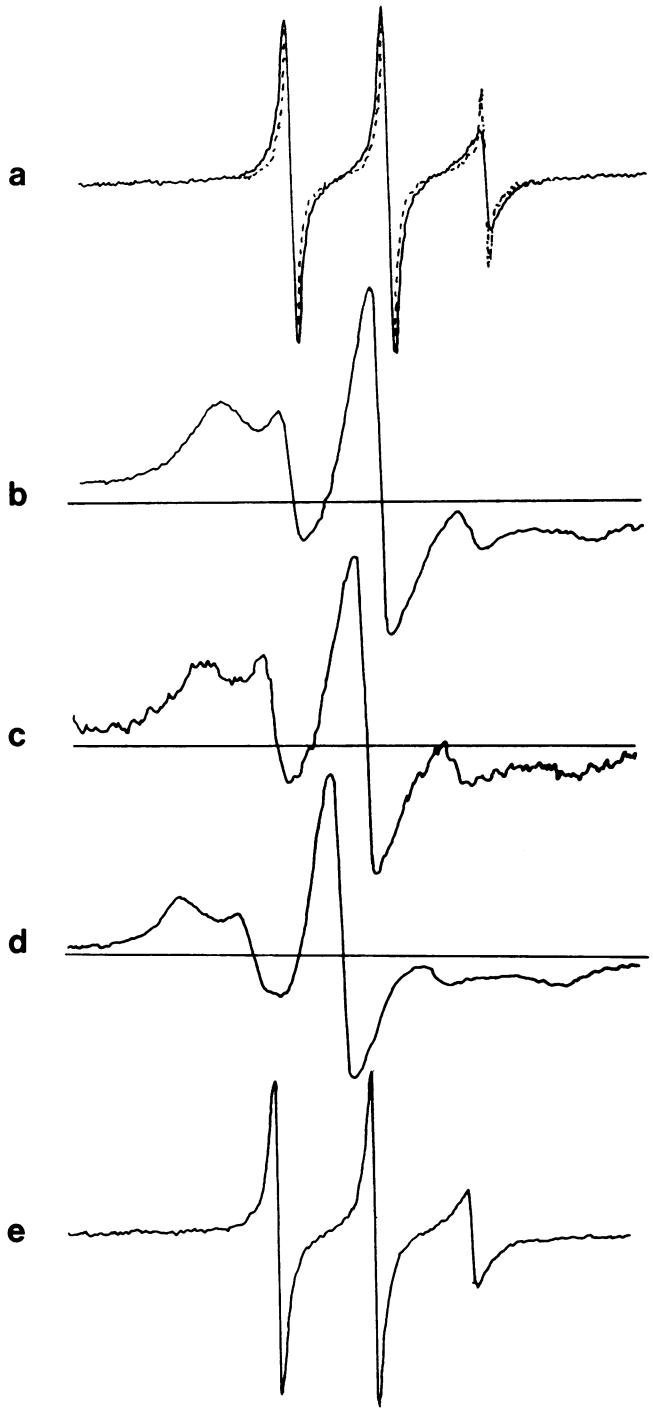

Fig. 1. E.s.r. spectra of spin-labelled α-bungarotoxins. **(a)** (full lines) double-labelled toxin (2 ^{2}H,MSL/mol toxin) in solution; (dotted lines) single-labelled toxin (1.2 ^{2}H,MSL/mol toxin) in solution. **(b)** Double-labelled toxin bound to ACh-receptor rich membranes. **(c)** Double-labelled toxin bound to the membranes half reacted with unlabelled toxin. Note that spectra **c** and **d** correspond to an immobilized spectrum superimposed to a broad wave revealing spin-spin interactions. The wave is barely seen in spectrum **d.** **(d)** Single-labelled toxin bound to membranes. **(e)** Single-labelled toxin in the presence of membranes saturated with unlabelled α-toxin; or labelled toxin displaced from the membrane by 1 M carbamylcholine.

the ACh-receptor sites.

The strong immobilization of the double-labelled toxin, which took place upon binding to the ACh-receptor site, made it a useful probe to follow the rotational diffusion of the toxin-receptor complex by ST-e.s.r. However, the presence of spin-spin interaction was accompanied by an almost complete loss of the 90° second harmonic signal. The saturation transfer measurements were thus performed with a single-labelled α-bungarotoxin. This toxin was obtained by the alkylation procedure 2 [50% ^{2}H,MSL + 50% N-ethyl maleimide (NEM)] described in Materials and methods. The average labelling ratio was 1.2 ^{2}H,MSL per toxin molecule and no further purification of the toxin was performed. The spectrum of the "single-labelled" toxin is presented in Figure 1a (dotted line): it gave narrower lines than the double-labelled toxin (full line) indicating that a small amount of spin-spin interaction (although not evident *a priori*) did exist in the soluble form of the biradical.

The spectrum of the single-labelled toxin (1d) bound to the membranes disclosed both a strong immobilization of the probe and much less interaction between spin labels than the double-labelled toxin. Again this remaining interaction was still observed at low occupancy of the toxin sites confirming that it was intramolecular (1d) and most likely resulted from a minimum contamination of the preparation by 10% of double-labelled toxin. The strong immobilization of the label and the decreased spin-spin interaction made this single-labelled α-bungarotoxin a convenient probe to follow the

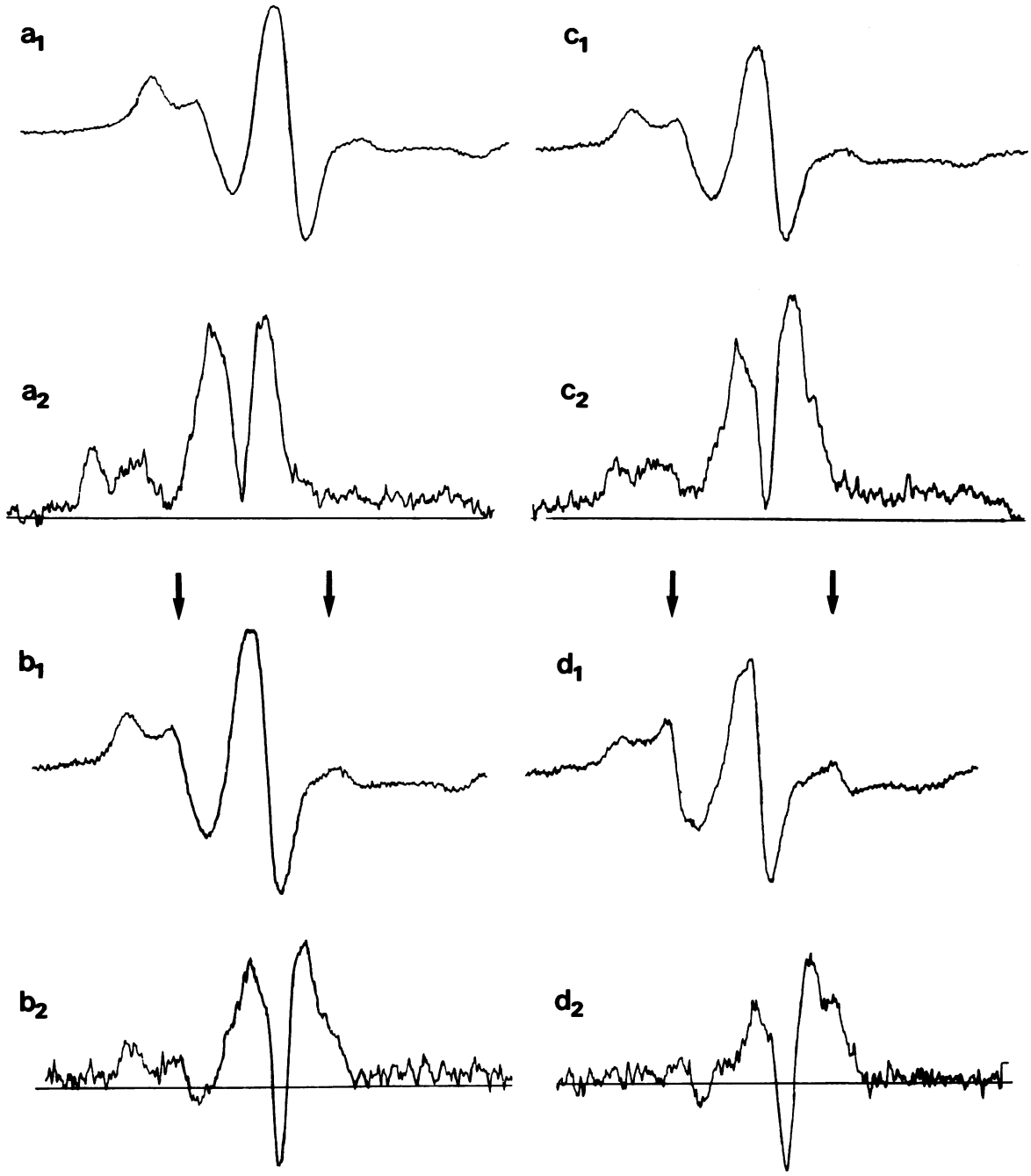

Fig. 2. First harmonic in phase spectra (a_1, b_1, c_1, d_1) of single-labelled α-bungarotoxin bound to the ACh-receptor rich membranes and the corresponding second harmonic out of phase spectra (a_2, b_2, c_2, d_2). a_1, a_2 native membranes, b_1, b_2 membranes treated at pH 11. c_1, c_2 and d_1, d_2 native and pH 11 treated membrane reduced by DTT.

rotational mobility of the membrane-bound ACh-receptor.

Rotational diffusion of the membrane-bound ACh-receptor after pH 11 extraction and/or treatment with DTT

Figure 2 shows the conventional e.s.r. spectra (first harmonic in phase) and ST-e.s.r. spectra (second harmonic, 90° out of phase) of native (Figure $2a_1$, $2a_2$) and pH 11 extracted (Figure $2b_1$, $2b_2$) membranes. No difference was noticed between first harmonic spectra (Figure $2a_1$, $2b_1$) which indicated that the labelled toxin bound to the receptor protein as rigidly in the native membranes as in the pH 11 extracted ones. On the other hand, the ST-e.s.r. spectra of the two categories of membranes differed strikingly. The spectra resembled those previously reported between the same samples but after direct labelling of the membranes with MSL instead of the toxin (Rousselet *et al.*, 1979b, 1981). Under the present conditions, a highly specific labelling was obtained; however, the rather low specific activity of the membranes prepared in the presence of anti-proteolytic agents (spin-label concentration 25 μM) and residual spin-spin interaction resulted in a low signal-to-noise ratio which did not allow exact determination of rotational correlation times. Nevertheless, the differences were striking enough to give orders of magnitude of rotational correlation time: $0.5-1 \times 10^{-3}$ s with the native membranes (spectrum $2a_2$) and $0.5-1 \times 10^{-4}$ s with the alkaline treated ones (spectrum $2b_2$). These time ranges were close to the correlation times previously reported with the maleimide-labelled membranes (Rousselet *et al.*, 1981), although in both instances slightly faster, possibly because of the larger distance existing between the spin label and the receptor protein. In any case, these data clearly showed that removal of the 43 000

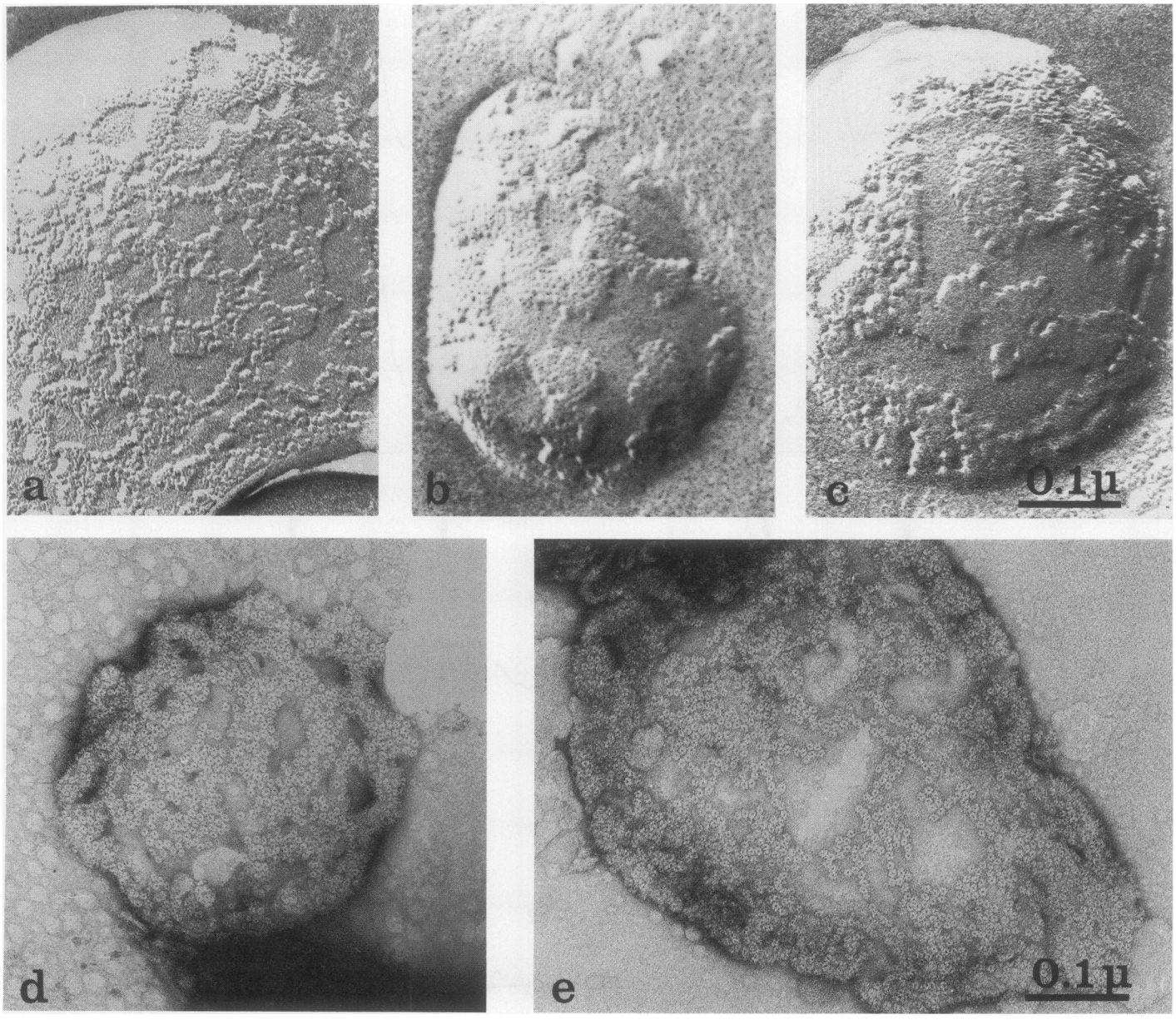

Fig. 3. Electron micrographs of alkaline extracted and DTT or β-mercaptoethanol reduced ACh-receptor rich membrane. **(a)** Freeze-etched view of pH 11 treated membrane. Note the reticular aspect of the distribution of the receptor rosettes at the membrane surface. **(b)** and **(c)** Freeze-etched aspect of a membrane fraction reduced by 20 mM β-mercaptoethanol: no comparable reticulation occurred upon freezing. **(d)** Negatively-stained membranes reduced by DTT after fusion with phosphatidylcholine vesicles. Clusters or file of rosettes are visible. Comparable images are obtained with unreduced native membrane. **(e)** x 160 000.

protein(s) by pH 11 extraction enhanced the rotational diffusion of the ACh-receptor protein at 20°C and not exclusively after heating at 39°C as recently claimed by Bartholdi *et al.*, (1981).

The same authors have reported that treatment of the native membranes by the disulphide bond breaking agent, DTT, enhanced the rotational diffusion of the receptor in a more significant fashion than pH 11 treatment (Bartholdi *et al.*, 1981). In our previous work (Rousselet *et al.*, 1979b, 1981), the attachment of the MSL was carried out after reduction by DTT. The consequence of DTT treatment on the rotational diffusion of the receptor protein was thus reinvestigated with the spin-labelled toxin. Native and pH 11 treated membranes were reduced by DTT, alkylated by NEM and then exposed to the paramagnetic toxin. A decrease of ~50% of the amount of toxin bound to the membranes took place under these conditions, but the first harmonic ($2a_1$, $2c_1$) and the second harmonic out of phase ($2a_2$, $2c_2$) spectra given by the native membranes did not significantly differ before ($2a_1$, $2a_2$) and after ($2c_1$, $2c_2$) treatment by DTT. On the other hand, in the alkaline extracted membranes, a slight difference in the first and second harmonic spectra ($2d_1$, $2d_2$) appeared, after reduction by DTT. The difference between $2b_2$ and $2d_2$ suggests a minor increase in the motion of the cholinergic receptor in the direction expected from the conversion of the receptor protein from its heavy (500 000 dalton) to its light (250 000 dalton) form known to occur under these conditions Sobel *et al.*, 1977; Change and Bock, 1977; Hamilton *et al.*, 1977). However, the rather low signal-to-noise ratio prevented the measurement of the expected variation of the correlation time (i.e., of the order of a factor of 2). In any case, the data showed that alkaline extraction enhanced the motion of the membrane-bound receptor in a much more significant manner than reduction by DTT.

Electron microscopy

Under conditions of slow freezing (as usually achieved with the conventional Balzers specimen holder dipped in liquid freon 22), a lateral redistribution of the receptor rosettes consistently occurred with the ACh-receptor rich membranes after alkaline extraction (Figure 3 and Cartaud *et al.*, 1981). Such a redistribution was never observed with native membranes or after treatment with 20 mM β-mercaptoethanol (Figure 3b and c). Observations made after negative staining confirmed these results. When the membranes were fused with phosphatidylcholine vesicles, a dispersion of the rosettes took place after alkaline extraction but never before (Cartaud *et al.*, 1981, Rousselet *et al.*, 1981). On the other hand, after treatment by DTT, the lipid-fused membranes never displayed scattered rosettes or doublets but rather, as in the case of the native membranes, clusters or files of rosettes interspaced by smooth lipid areas (Figures 3d and 3e).

Discussion

To follow accurately the rotational diffusion of a large macromolecule such as the ACh-receptor in its membrane-bound state, a probe showing complete immobilization upon binding to the receptor is required. Several spin-labelled toxins have thus been synthesized. The results of Tsetlin *et al.* (1979), Ellena and McNamee (1980), and Faure, Rousselet, Boulain, Deraux, Fromageot, and Menez (unpublished data) show that most of the toxins labelled on ϵNH_2 of lysine residues give only partially immobilized signals upon binding to the ACh-receptor. Among the toxins labelled on their -SH group by the method of Chicheportiche *et al.* (1975) only one of the four species separated by h.p.l.c. showed immobilization (Rousselet and Fellman, unpublished results). On the other hand, the α-bungarotoxin labelled by the method of Botes (1974) yielded completely immobilized signals and was thus selected for the e.s.r. experiments. In addition, the spectrum of this double-labelled toxin exhibited large spin-spin interaction after attachment to the ACh-receptor. Since this interaction was barely seen in the unbound state of the α-toxin, a change of conformation, which would bring the two spin-labelled cysteines of the toxin close to each other, probably took place upon binding to the ACh-receptor site.

Since this spin-spin interaction interfered with the measurements by saturation transfer, a derivative carrying only one spin label was used to probe the rotational diffusion of the toxin receptor complex in isolated membrane fragments. The ST-e.s.r. spectra confirmed (Rousselet *et al.*, 1979b, 1981) that alkaline extraction which eliminated peripheral proteins, and in particular the internal 43 000 polypeptide(s), from the receptor-rich membranes markedly enhanced the rotational diffusion of the receptor protein. On the other hand, treatment of the membranes by the disulphide bond reducing agent DTT did not show this effect but only a slight distortion in the central part of the ST-e.s.r. spectra. Electron microscopy also showed that DTT had little, or no, effect on the redistribution of the receptor rosettes compared with the alkaline treatment. In contrast, Bartholdi *et al.* (1981) using phosphorescence depolarisation did not see any effect of the alkaline treatment at room temperature but reported an increased motion of the probe only after heating the alkali stripped membranes or after exposure to DTT. A plausible interpretation of the difference is that these authors have not followed the rotational motion of the receptor-toxin complex but rather the segmental movement of the toxin probe on the receptor protein. The low value that Bartholdi and collaborators report for the rotational correlation time with their eosin thiocyanate-labelled toxin is consistent with this interpretation. Indeed, as already mentioned, a mixture of toxin spin-labelled on the ϵNH_2 lysine residues may not give completely immobilized spectra upon binding to the receptor. Using the same technique as Bartholdi *et al.* (1981) but a different labelled toxin, Lo *et al.* (1980) have reported an enhanced motion at 2°C of the receptor after pH 11 treatment.

In conclusion, protein-protein interactions appear primarily responsible for the immobilization of the ACh-receptor in the post-synaptic membrane. As suggested (Saitoh *et al.*, 1979; Rousselet *et al.*, 1979b, 1981; Barrantes *et al.*, 1980), these interactions may play a critical role in the formation of the sub-synaptic membrane during embryonic development (reviewed by Changeux, 1981).

Materials and methods

Preparation of ACh-receptor rich membranes

Membrane fragments were prepared from fresh electric organs from *T. marmorata*. Homogenization of the tissue and the following centrifugation steps were performed in the presence of anti-proteolytic agents as described by Saitoh and Changeux (1980). The fish were provided by the Laboratoire Arago, Banyuls/mer and the Station de Biologie Marine, Arcachon, France. Specific activity of the membrane fragments was determined by using either a spin-labelled α-bungarotoxin or an [^{3}H]α-toxin from *Naja nigricollis*, a generous gift from A. Menez, CEN, Saclay, France. The average specific activity of the ACh-receptor rich membranes was 1500 nmol of α-toxin binding sites/g of protein.

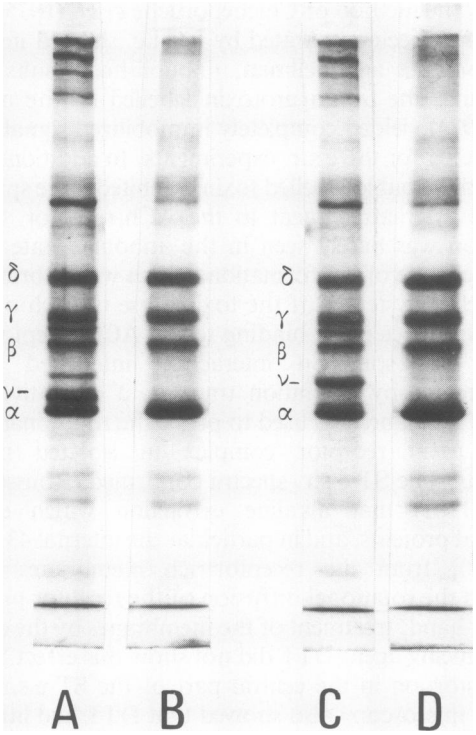

Fig. 4. Polyacrylamide gel electrophoresis of ACh-receptor rich membranes performed in the presence of SDS as described by Sobel *et al.* (1977). **(A)** Native membranes. **(B)** Alkaline treated membranes. **(C)** Native membranes prepared in the presence of 5 mM NEM. **(D)** Alkaline treated membranes prepared in the presence of NEM. Note in both cases the complete extraction of the ν peptides. When membrane fragments are prepared in the presence of NEM the ν peptide(s) migrate more slowly in the SDS gel (Barrantes, 1982).

Membrane modifications

The pH 11 extraction (Neubig *et al.*, 1979) was performed during 30 min at 0°C as described by Rousselet *et al.* (1979b). Complete reduction of the disulphide bridges of the ACh-receptor was achieved with 1 mM DTT at pH 8.6 in the presence of 3 mM EDTA during 45 min at room temperature. These membranes were then alkylated with 10 mM NEM overnight at 4°C. All these reactions were performed with buffers saturated with argon and under an argon atmosphere. Unreacted products were washed away by centrifugation in *Torpedo* Ringer solution (0.5 M NaCl, 10 mM KCl, 1 mM EDTA, 50 mM Tris pH 7.5). The resulting membranes were resuspended in the same buffer for labelling by paramagnetic α-bungarotoxin. Protein composition of the receptor rich membranes was followed by SDS gel electrophoresis (Figure 4).

Labelling of α-bungarotoxin by deuterated 2,2,6,6 tetramethylpiperidinooxyl: ^{2}H,MSL

The paramagnetic maleimide bears deuterium instead of hydrogen on its four methyl groups. It was a generous gift from L. Dalton, Department of Chemistry, State University of New York at Stony Brook, NY. Reduction of the additional disulphide bridge of the long neurotoxin (bungarotoxin) was performed as described by Botes (1974). The reduced α-bungarotoxin was then alkylated in two ways: (1) with a 10-fold excess of pure ^{2}H,MSL; or (2) with a mixture of a 5-fold excess of ^{2}H,MSL plus a 5-fold excess of NEM. After overnight incubation, excess reactives were eliminated by a column of Sephadex G 50 fine in 50 mM ammonium carbonate. Homogeneity of the paramagnetic toxin prepared by alkylation (1) was checked by h.p.l.c. Quantification of the labelling was carried out by integration of the e.s.r. signal. The absolute concentration of spin label on the toxin molecules was obtained by comparing this integration with a reference sample. Protein was titrated by optical density measurements and by the Lowry technique using serum albumin as a standard.

Labelling of the ACh-receptor in its membrane-bound state

Membrane solutions (5 mg/ml protein) were incubated in the presence of a 3-fold excess of paramagnetic α-bungarotoxin. After 20 min incubation at 4°C, the unreacted toxin was washed by two series of centrifugations in *Torpedo* Ringer solution. The specificity of the labelling was tested by incubating the membranes with 5-fold excess of unlabelled α-toxin followed by washing and addition of the labelled α-toxin. Specific dissociation of the labelled α-toxin was also tested by adding 1 M carbamylcholine to membranes labelled with the paramagnetic toxin.

Electron microscopy and e.s.r.

These were performed under the same conditions as those described in Cartaud *et al.* (1981) and in Rousselet *et al.* (1981). The rotational correlation time was deduced from the ST-e.s.r. spectra using the procedure of Thomas *et al.* (1976). The use of deuterated spin label ^{2}H,MSL allowed a better e.s.r. signal resolution and a better sensitivity. Deuterium abolished the superhyperfine interactions of the methyl groups with the unpaired electron of the nitroxide, giving spectra with sharper lines (Beth *et al.*, 1980).

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