ROTATIONAL MOBILITY OF THE MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR OF *TORPEDO* ELECTRIC ORGAN MEASURED BY PHOSPHORESCENCE DEPOLARISATION

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1. Introduction

The molecular organisation of the ACh receptor protein in its in situ state has been extensively studied in receptor-rich membranes prepared from fish electric organ, using several different techniques. A hexagonal lattice array of this receptor was deduced from X-ray diffraction data [1,2] and from some freeze-fracture [3,4] or negative staining [1,3,4] studies, but not some others [5–7]. Thus, in the freeze-etched, rotary-shadowed replicas obtained from fresh, rapidly-frozen *Torpedo* electrocytes [7], a close-packed lattice was not seen, but rather particles mainly in loosely-packed irregular double rows or random aggregates that might leave the possibility of some rotational mobility of the individual receptors. The method of saturation transfer EPR has also been applied to these membranes, to study the rotational mobility of the ACh receptor (reacted with a spin-labelled maleimide) [8]. The ACh receptor was reported thus to show no rotational motion [8] over a time-range of up to 1 ms. It was also deduced from other EPR studies [9,10] that some fatty acids and steroids can be immobilised near, or on [10], the ACh receptor protein; in contrast, EPR has also indicated that the lipid phase around the receptors is fluid [10,11].

Abbreviations: ACh, acetylcholine; ErITC, erythrosin isothiocyanate; BuTX, α-bungarotoxin; ErBuTX, α-bungarotoxin labelled covalently with erythrosin; [^{3}H]BuTX, mono-[^{3}H]-propionyl-α-bungarotoxin; EPR, electron paramagnetic resonance spectroscopy

Hence, confirmation by an independent method of the receptor protein rotational immobility is desirable.

Furthermore, saturation transfer EPR is a relatively insensitive method of measuring rotational diffusion, and cannot be applied to other receptors, which generally occur in much lower amounts. For example, the ACh receptors in skeletal muscles are present in suitable membrane fractions at levels $\sim 10^{-3}$-times lower (for denervated muscles [12]) than that found in purified electric organ membrane (~2 nmol BuTX binding sites/mg membrane protein [13]), and much lower still in membranes from normal innervated muscles. The muscle receptor may be either entirely concentrated at the synapse, as in normal adult fast-twitch muscle, or be diffusely distributed over the surface of the whole muscle fibre, as in denervated or embryonic muscles, and hence might vary in its mobility.

A phosphorescence depolarisation method for measuring rotational mobility of membrane proteins has been developed [14–16] (see also [17]). This new method uses erythrosin (tetra-iodofluorescein) as the phosphorescent probe, is $\sim 10^4$-times more sensitive than saturation transfer EPR, and provides an opportunity to study the rotational motion of proteins present in the membrane at low concentrations. Here we describe the application of this method, using the specific irreversible binding of BuTX, to the ACh receptor. We provide initial, independent confirmation of the immobility of the ACh receptors in the *Torpedo* electric organ membrane. Extraction of peripheral membrane proteins with alkali [18–20] is shown to cause considerable loss of this immobility.

2. Methods

2.1. *Preparation and activity of ErBuTX*

Erythrosin (tetra-iodofluorescein) isothiocyanate (ErlTC) was synthesised as in [14]. BuTX was purified from venom of *Bungarus multicinctus* [21]. BuTX (4 mg) was treated with ErlTC (0.34–3.40 mg) in 0.5 ml 50 mM $NaHCO_3$ (pH 9) for 0.5 h at room temperature in the dark. Residual reagent was removed by gel filtration on a Sephadex G-25 column (1.1 × 20 cm) in 50 mM ammonium acetate (pH 5). Erythrosin incorporation was determined at 540 nm using $\epsilon = 83\ mM^{-1}cm^{-1}$; protein determination [22] used BuTX as standard.

To determine the binding activity on the receptor, 1.4 pmol (on a total toxin basis) of ErBuTX was reacted with 8 pmol freshly-solubilised [22] receptor-rich membranes. Receptor concentrations were determined in assays [22] by [^{3}H]BuTX binding on aliquots of the receptor preparation. The [^{3}H]BuTX (50 Ci/mmol) was prepared and purified as in [23]. The reaction with ErBuTX went to completion (15 h at 2°C, in 0.4 ml 50 mM Na-phosphate buffer (pH 8)/0.2% Triton X-100). The reaction mixture, and parallel samples of receptor with ErBuTX omitted (as the control), were then assayed for free receptor content using [^{3}H]BuTX. This showed 90% blockade of the initial receptor present had occurred. Dissociation of the soluble ErBuTX–receptor complex was measured in the presence of 10-fold excess [^{3}H]BuTX at 22°C, assaying [22] the [^{3}H]BuTX–receptor complex formed after 4 h.

2.2. *Preparation of receptor-rich membranes*

ACh receptor-rich membranes were prepared from both fresh and frozen *Torpedo marmorata* electric organ (the latter stored at −70°C) by density gradient centrifugation [13]. Membranes obtained from the discontinuous gradient routinely contained 1.0–1.5 nmol [^{3}H]BuTX binding sites/mg protein. For alkaline extraction, crude and purified membranes were treated at pH 11 [18], for (2 × 1 h) at 2°C, without loss of receptor activity. Membranes were finally resuspended in 50 mM Na-phosphate buffer (pH 7) and reacted with an amount of ErBuTX equal (on a toxin molar basis) to half of the ACh receptor present, at 2°C for 15 h in the dark.

2.3. *Measurement of phosphorescence depolarisation*

Membrane samples (~1.0 mg membrane protein/ml) were diluted with an equal volume of 98% (w/v) glycerol (final vol. 1–2 ml), and de-aerated under a stream of Argon gas for 15–20 min [15]. Laser flash-excited phosphorescence of erythrosin was measured [16] using a Phase-R model DL-1200 flashlamp-pumped dye laser operating with 1.5×10^{-4} M Couramin 6 in methanol, delivering to the sample ~1 mJ at 540 nm, at a 1 Hz repetition rate. Separate photomultipliers protected by red filters (Schott RG695, 3 mm) measured the phosphorescence emission polarised either parallel (∥) or perpendicular (⊥) to the plane of polarisation of the excitation flash. A Nicolet 1170 twin channel signal averager stored 16, 32 or 64 sweeps corresponding to the (∥ − ⊥), and (∥ + 2 ⊥) signals, and also computed the anisotropy parameter r [24], which is (∥ − ⊥)/(∥ + 2 ⊥). The relationship between the time dependence of the decay of r and the rotational diffusion of the molecule under study are discussed in depth in [25]. The substantially positive value of r for fully immobilized erythrosin [15] suggests that the transition moments for absorption and phosphorescent emission are parallel. We therefore assume that the time dependence of r is given by:

$$r(t) = A_1\, e^{-Dt} + A_2\, e^{-4Dt} + A_3 \qquad (1)$$

where: $A_1 = (6/5)(\sin^2\theta\cos^2\theta)$, $A_2 = (3/10)(\sin^4\theta)$, $A_3 = (1/10)(3\cos^2\theta - 1)^2$; D is the rotational diffusional coefficient of the probe-carrying protein about an axis normal to the plane of the membrane; and θ is the angle between that axis and the transition moment for absorption [25]. The anisotropy parameter r becomes independent of time either when D^{-1} is large relative to the duration of observation, or when $\theta = 90°$ (whereupon $r = A_3$). If there are two triplet components contributing to the phosphorescence with lifetimes τ_1 and τ_2, then the time-dependent anisotropy of the total anisotropy is given by:

$$r(t) = \frac{a_1 r_1(t) e^{-t/\tau_1} + a_2 r_2(t) e^{-t/\tau_2}}{a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}} \qquad (2)$$

where a_1 and a_2 are the phosphorescent intensities of the two components at zero time, and r_1 and r_2 their respective time-dependent anisotropies [26].

2.4. *Negative staining of membranes*

Aliquots (25 μl) of purified membranes (0.1 mg

protein/ml, without fixation treatment) before and after alkaline extraction, with bacitracin (10 μg/ml) added, were deposited on carbon-coated grids. The grids were blotted and aqueous uranyl acetate (1%, pH 4.5) was applied. The air-dried grids were examined in a Philips 301 electron microscope, calibrated using the catalase crystal lattice [27].

3. Results and discussion

3.1. *Phosphorescent derivative of BuTX*

BuTX was reacted with erythrosin isothiocyanate to give a covalently-modified phosphorescent toxin. After reaction at varying ratios of reagent:BuTX from 1–10, the excess reagent was separated by gel filtration, to yield a protein fraction containing from (respectively) 0.6–2 bound erythrosin groups/BuTX molecule. The lowest of these degrees of conjugation was selected for membrane labelling, to ensure that depolarisation due to triplet energy transfer was minimised in the labelled membranes.

The derivative used contained, therefore av. 0.6 erythrosin groups/BuTX molecule, being mainly a mixture of mono-labelled (60%) and native (40%) BuTX species, since the starting molar amounts of the reagent and toxin were equal. The ErBuTX was shown to be fully active as a receptor binding toxin, in that after reaction to completion with 5 equiv. of the ACh receptor in solution, the binding of [^{3}H]BuTX to the latter was completely blocked (see section 2).

Subsequent incubation at room temperature of the complex formed thus of ErBuTX with the receptor, up to 4 h in the presence of excess [^{3}H]BuTX, showed that no dissociation of the derivative occurs in this time, since no [^{3}H]BuTX binding was restored thus. Hence, this toxin derivative (ErBuTX) binds essentially irreversibly, and all the sites of its phosphorescent labelling are ACh receptors.

3.2. *Phosphorescence of ErBuTX labelled* Torpedo *membranes at 2°C*

Polarised phosphorescence signals arising from laser flash-excited ErBuTX-labelled membranes at 2°C are shown in fig.1. The recordings given are for the ($\parallel - \perp$), ($\parallel + 2\perp$) and log ($\parallel + 2\perp$) signals. The ratio ($\parallel - \perp$)/($\parallel + 2\perp$) is also plotted, being the anisotropy parameter *r*. There are several noteworthy features in these recordings:

(i) There is an excellent signal-to-noise ratio despite the low concentration of ErBuTX (5×10^{-7} M);

(ii) The decay of the phosphorescence, given by log ($\parallel + 2\perp$), is not a single exponential process, there being at least two components with lifetimes of ~200 μs and 500 μs.

(iii) The anisotropy parameter remains between 0.09 and 0.10 for up to 900 μs after the laser flash. Beyond 900 μs the signal-to-noise ratio increases markedly as the phosphorescence decays further.

Nevertheless, it can be concluded that any rotation of the ACh receptor at 2°C must have a rotational correlation time of several ms or more.

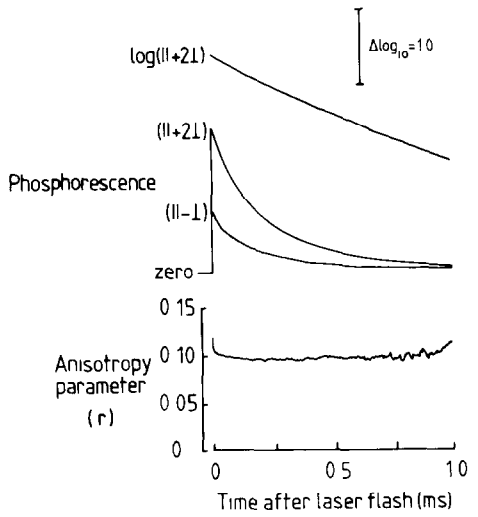

Fig.1. Phosphorescence of ErBuTX-labelled membranes at 2°C. The sample (2 ml) contained 0.83 mg membrane protein (0.98 nmol [^{3}H]BuTX-binding sites) labelled with 0.5 nmol ErBuTX (total BuTX content). Excitation was with 32 laser flashes, each of 1 mJ. The initial fast decrease in the anisotropy parameter *r* occurs within 5 μs of the laser flash, and is due to artefacts associated with the laser flash.

3.3. *Alkaline-extracted* Torpedo *membranes: phosphorescence depolarisation*

It has been shown [18] that extraction at pH 11 in low ionic strength media of *Torpedo* receptor-rich membranes removes a major 43 000 dalton polypeptide and some other proteins, but none of the receptor. Moreover, these depleted membranes retain also the specific binding sites for local anaesthetics and histrionicotoxin and the ability to exhibit or recover agonist-dependent Na^+ flux, these properties testifying to the presence of the synaptic ion channel of the receptor [18–20]. We have, therefore, used

this form of the *Torpedo* membrane with a simplified constitution, which should lack non-membrane or peripheral proteins [28], to study any possible increase in the mobility of the receptor. Fig.2. shows recordings of the phosphorescence [log ($\parallel + 2\perp$)] and r for normal and alkali-extracted membranes at 2°C and 26°C. The behaviour of the normal membranes at 2°C was commented on above; at 26°C the phosphorescence decay was faster, especially in the case of the more rapidly decaying component. As at 2°C, the anisotropy at 26°C did not fall below 0.1, but if anything showed a slight time-dependent increase, not usually as marked as in the example given. The behaviour of the alkali-extracted membranes was quite different:

(i) the phosphorescent lifetimes were shortened, especially that of the faster component, which was reduced by a factor of 3–4 fold;

(ii) r fell to a low value of ~0.07 within 20 μs of the laser flash, then increased slowly to ⩾0.1. The duration of the phase of increasing anisotropy appeared to match that of the fast phase of phosphorescent decay.

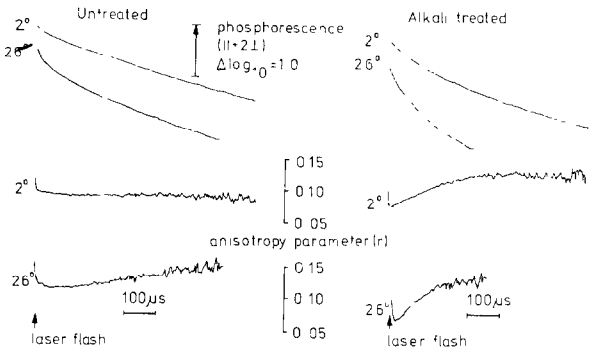

Fig.2. Effects of alkali-extraction on phosphorescence of ErBuTX-labelled membranes at 2°C and 26°C. Conditions were otherwise as for fig.1, except that the protein concentration for the alkali-extracted sample was halved. Traces for untreated membranes are on the left and for alkali-extracted membranes on the right. The relative zero-time contributions of the faster and slower decaying components of the phosphorescent traces were estimated by extending the slower linear part of the log ($\parallel + 2\perp$) trace back to zero time, and then calculating the ratio of the total phosphorescence to the estimated slow phosphorescent component from the difference in their logarithms at zero-time. In an additional experiment the alkali-extracted, labelled membranes after showing this behaviour were extensively crosslinked (as proven by SDS gel electrophoresis) by dimethylsuberimidate treatment; when excited then, the time dependence of r was as shown here for the untreated membranes at 2°C, i.e., all the change in r was abolished.

The interpretation of phosphorescence polarisation decay curves is complicated when there are two or more components present with markedly different lifetimes [26]. Nevertheless, a model consistent with both eq. (2) and our observations would assign a low value of r to the faster decaying component and a high value (~0.1–0.125) to the slower decaying component. There are two possibilities for the low anisotropy of the faster component: either the environment has brought the transition moment for emission to lie at a large angle relative to that for excitation, or there has been fast rotation within about 20 μs. Since we have never observed a value of $r < 0.09$ for erythrosin immobilized in a wide variety of conditions, we discount the first possibility and conclude that the initial low anisotropy in the alkali-treated membranes is due to fast rotation of the more rapidly decaying component which accounts for ⩾70% of the total phosphorescence at zero time. A more detailed examination of the first 10–20 μs after the laser flash must await improved instrumentation. However, strong support in favour of our conclusion that alkali extraction permits rotation of the ACh receptor comes from our further observation that after chemical crosslinking the alkali-extracted membrane exhibited a time-independent anisotropy parameter similar to that of the normal membrane (see legend to fig.2).

3.4. *Alkali-extracted* Torpedo *membranes: protein composition and electron microscopy*

In the alkali-extracted membranes used, SDS gel electrophoresis (performed as in [18,20]) showed prominently the presence of the subunits in that system of ~40 000 (the strongest), 50 000, 58 000 (very weak) and 66 000 daltons which are found in the purified ACh receptor protein from *Torpedo*, in good agreement with [18–20]. The band at 43 000 daltons, which was very strong in the native membranes, was completely absent in the extracted membranes, but was recovered in the extracting supernatant, and some other weaker bands were likewise removed and recovered, again as in [18–20]. The prominent band at ~92 000 daltons, attributed to the major subunit of Na^+,K^+-ATPase [18], was still present in the extracted membranes. The protein content of the membranes used was reduced by ~50% in the base extraction, whereas the receptor activity was unchanged. It was of interest, therefore, to examine these membranes also for their ultra-

structural organisation. In negatively-stained electron micrographs (fig.3a) the characteristic rosettes of the native membrane [3–6] were seen. In the alkali-extracted membranes shown here, these rosettes remained unchanged in their morphology (fig.3b). It was also seen that the native membrane existed mainly as large sheets, whereas the extracted membranes were mainly small vesicles, ~0.05–0.5 μm diam. The image due to the stained protein in this membrane was sharper after the base extraction than hitherto seen, and it was clear that in these unfixed membranes the rosettes (interpreted as the receptor-ion channel assemblies [4–7]) are not close-packed but are separated structures, not in a regular lattice, despite their high density in the membrane. This is in agreement with the conclusion drawn from a study of quick-frozen freeze-etched specimens [7] and contrary to results with negative staining [3,4]. The rosettes when seen in the extracted membranes (fig.3b) had a doughnut-shaped profile, with a densely stained centre (indicating a well [1]). The mean (± SD) external diameter was 68.1 (± 6.7) Å before extraction, and 70.8 (± 6.9) Å after, i.e., without significant change. The density was 6600 μm^{-2} before extraction, and 9400 μm^{-2} after; the latter value is in excellent agreement with the density of ~10 000 μm^{-2} for the corresponding projections on the external face of the native *Torpedo* synaptic membrane that were seen in the quick-frozen freeze-etched preparation [7].

Hence, the removal of the 43 000 dalton and some other protein by alkali extraction does not change the prominent features of the organisation of the receptor in the membranes as judged by electron microscopy, but it does permit rotation of the receptor with rotational correlation times of ⩽10–20 μs. Since all the functional properties of the receptor remain [18–20], we conclude that the synaptic ACh receptor can function whether mobile or not, and

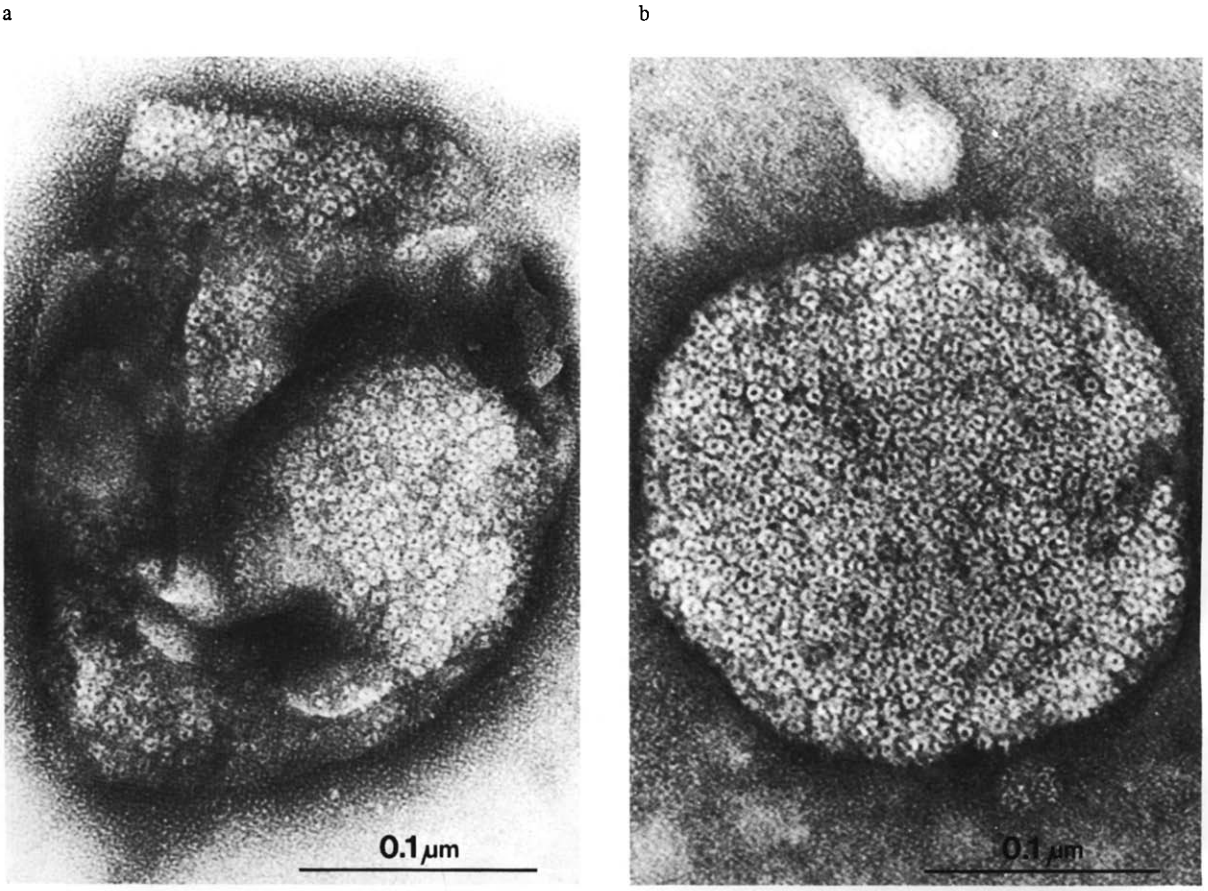

Fig.3. Negatively-stained *Torpedo* membrane fragments: (a) native; (b) after alkali extraction.

that interaction between neighbouring receptor assemblies is not the cause of the normal localisation. Rather, non-receptor components must normally anchor them. It seems, therefore, that an effect either of the 43 000 dalton polypeptide, or of some other peripheral protein removed at pH 11, is to immobilize the receptor assembly in post-synaptic patches.

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Note added in proof

Recently, A. Rousselet, J. Cartaud and P. F. Devaux (1979) Comptes Rendus 289, 461–463, have reported in preliminary form evidence obtained by an alternative approach which is in agreement with our conclusion. The receptors in *Torpedo* membranes were shown by saturation transfer EPR spectra to become mobile upon alkali extraction.

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