PEPTIDE EXTRACTION BY ALKALINE TREATMENT IS ACCOMPANIED BY REARRANGEMENT OF THE MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR FROM *TORPEDO MARMORATA*

F. J. BARRANTES, D.-Ch. NEUGEBAUER and H. P. ZINGSHEIM
Max-Planck-Institut für Biophysikalische Chemie, Göttingen-Nikolausberg, FRG

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1. Introduction

The ability to prepare membranes enriched in acetylcholine receptor protein (AChR) [1,2] constituted a significant contribution to in vitro studies of physiological significance (reviewed [3,4]). Work in this field has yielded evidence that the detergent-solubilized, purified AChR consists of subunits of 40 000 (α), 50 000 (β), 60 000 (γ) and 65 000 (δ) apparent molecular weight [3]. The M_r 40 000 peptide carries the recognition site for acetylcholine and its analogues, α-toxins, and affinity labels of the nicotinic type [3]. This subunit and a peptide of M_r 43 000 were reported to be the predominant components of the AChR-rich membranes [5,6]. More recently, it was suggested that the peptide of M_r 43 000 (the ν-band of [7]) carries the binding site for local anaesthetics and, by inference, the AChR-controlled channel [8]. Yet, sizeable amounts of the ν-component, together with some other peptides, can be extracted at alkaline pH leaving an AChR-rich membrane with normal agonist and local anaesthetic binding capacities; the permeability control of the $^{22}Na^+$ efflux is also retained [9]. The role of the above M_r 43 000 peptide remains therefore elusive.

A possible contribution of the M_r 43 000 peptide to the structure of the membrane has not yet been considered. The comparison of electron micrographs from untreated and alkaline-treated membranes yields significant differences in membrane organization, suggesting that the M_r 43 000 peptide may be important for stabilizing the AChR-rich membrane and the arrangement of the AChR protein in it.

Address correspondence to H.P.Z.

2. Material and methods

2.1. *Purification of the AChR-rich membrane fraction from* Torpedo marmorata *electric organ*

This was done essentially as in [1] or [5]. Protein content and specific activities of the resulting membranes were assayed as in [11], except that α-[^{3}H]-bungarotoxin (spec. act. 48 Ci/mmol, Amersham Buchler, Braunschweig) was used instead of α-[^{3}H]-cobrotoxin. Specific activities of the purest fractions were in the order of 2.5–3.5 nmol α-toxin/mg protein.

2.2. *Removal of peptides by alkaline treatment*

This was accomplished by incubating the AChR-rich membrane fragments at pH 11 as originally reported in [9]. Membrane stock suspensions in 37% (w/w) sucrose containing 0.02% NaN_3, 1 mM EDTA and 0.1 mM phenylmethane–sulphonylfluoride were adjusted to ~1 mg protein/ml and diluted 1:1 with distilled water. The pH was brought to 11 by addition of 1 μl aliquots of 1 N NaOH in a reacting vial under continuous stirring. Incubation at pH 11 was for 1 h at 20°C. The samples were then diluted in the above low-ionic strength medium and centrifuged for 45 min at 20 000 rev./min in a Sorvall SS-34 rotor at 4°C. The pellets were resuspended in the same medium containing 5 mM Na-phosphate buffer (pH 7.0) and the supernatant was concentrated in a Schleicher and Schüll dialysis bag under negative pressure.

2.3. *Polyacrylamide gel electrophoresis*

This was done as in [12], with the modifications indicated in the figure legends where appropriate. Protein at 10–30 μg was applied to each well on the 5% stacking gel polymerized over the 10% running gel.

Samples were dissolved in 2% SDS, 63 mM Tris–HCl buffer (pH 6.8) containing 10% sucrose and 0.0025% bromophenol blue, and boiled for 3 min in the presence of 100 mM dithiothreitol. Gel electrophoresis was performed in 0.15 × 14 × 18 cm slab gels at 10 mA/slab for 1 h and thereafter at 30 mA/slab for 5 h. Gels were stained overnight in 0.025% Coomassie blue and destained in 5% methanol, 7.5% acetic acid. Densitometry of the electropherograms was carried out in a Vernon scanner.

2.4. *Electron microscopy*

The membrane suspension, 2 μl (0.1–0.5 mg protein/ml) was put onto carbon-coated copper grids. After a few seconds the grids were rinsed with ~2 ml distilled water and stained with 1% uranyl formate.

The preservation of membrane specimens is (among other things) influenced by the surface properties of the support film [18]. Carbon films freshly prepared under the conditions in [18] are hydrophilic. After a few hours they become hydrophobic. Therefore specimens were prepared on fresh carbon films (0–2 h) as well as on old films (1–2 days).

Micrographs were taken with a Phillips EM 301 at 80 kV or with a high resolution scanning transmission electron microscope (STEM, Vacuum Generators Ltd.) at 60 kV. The centre to centre spacings of nearest neighbour particles were measured on micrographs (final magn. 500 000 ×) of areas with homogeneously packed particles (see fig.3, insets). Particle densities were determined by counting the particles on micrographs (final magn. 250 000 ×) of large membrane areas. The area values were determined gravimetrically.

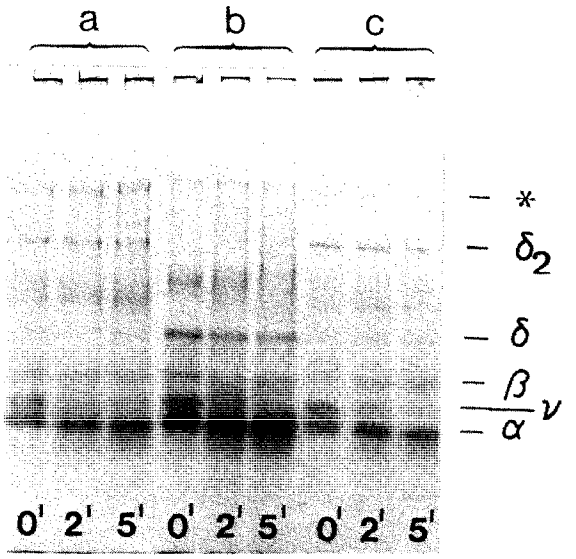
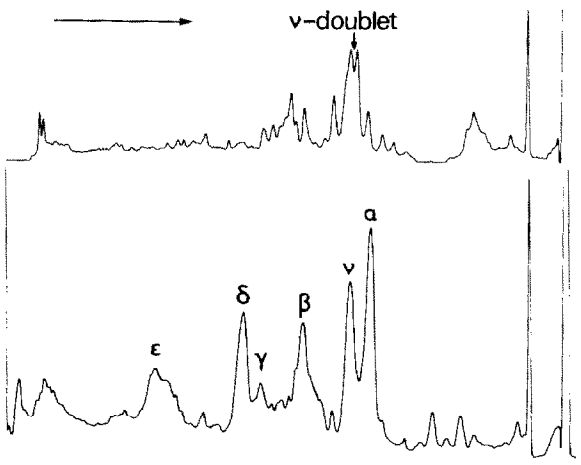


Fig.2. Influence of sample preparation on the gel electrophoresis polypeptide patterns. AChR-rich membranes from *T. marmorata* electric organ prepared according to [1] were solubilized in Laemmli's medium [12], containing: (a) neither oxidizing nor reducing agents; (b) 100 mM dithiothreitol; (c) 10 mM 5,5'-dithio-bis (nitrobenzoic) acid. The time prior to application of the samples to the stacking gel is indicated in each case; the '0 min' condition corresponds to the samples applied immediately after dissolution in the solubilizing mixture at 20°C. Slots labelled '2 min' and '5 min' correspond to the samples heated to 100°C for the indicated periods prior to their application to the gels. Note the progressive disappearance of the ν-band as a function of time for each set of samples and the concomitant appearance of low molecular weight material migrating in front of the ν-band. A high molecular component with an apparent electrophoretic mobility of M_r 200 000 (*) is most prominent in non-reduced samples (a,c).



3. Results

3.1. *The main component of the alkaline extract is a doublet (ν) of* M_r *43 000 on SDS gels*

Exposure of the AChR-rich membrane (microsacs) from *T. marmorata* to pH 11 at low ionic strength [9]

Fig.1. SDS–polyacrylamide gel patterns of untreated microsacs and the alkaline extract. Densitometric recordings of SDS–polyacrylamide gel electropherograms of 10 μg AChR-rich membranes from *T. marmorata* electric organ prepared according to [5] (lower trace) and of 25 μg of the pH 11.0 extract from the same [9] (upper trace). See section 2 for details. The arrow indicates the direction of migration.

results in an increase in the specific activity of the membrane fraction between 40–60% (in terms of α-[^{3}H]bungarotoxin sites). This increase in purity can be accounted for by the relative enrichment of the α-component (M_r 40 000) at the expense of other polypeptides migrating with app. M_r ~43 000 (*ν*), 55 000 and 60 000 (fig.1), as shown in another species of *Torpedo* [9]. The dominating band in the alkaline extract is a doublet with an apparent electrophoretic mobility of M_r ~43 000, which normally appears as a single band in samples of untreated microsacs because of the usual overloading of the gel (cf. fig.1,2).

3.2. *The intensity of the ν-band depends critically on sample conditions*

The *ν*-band shows up most conspicuously in samples from native membrane preparations applied to the stacking gel immediately after solubilization at

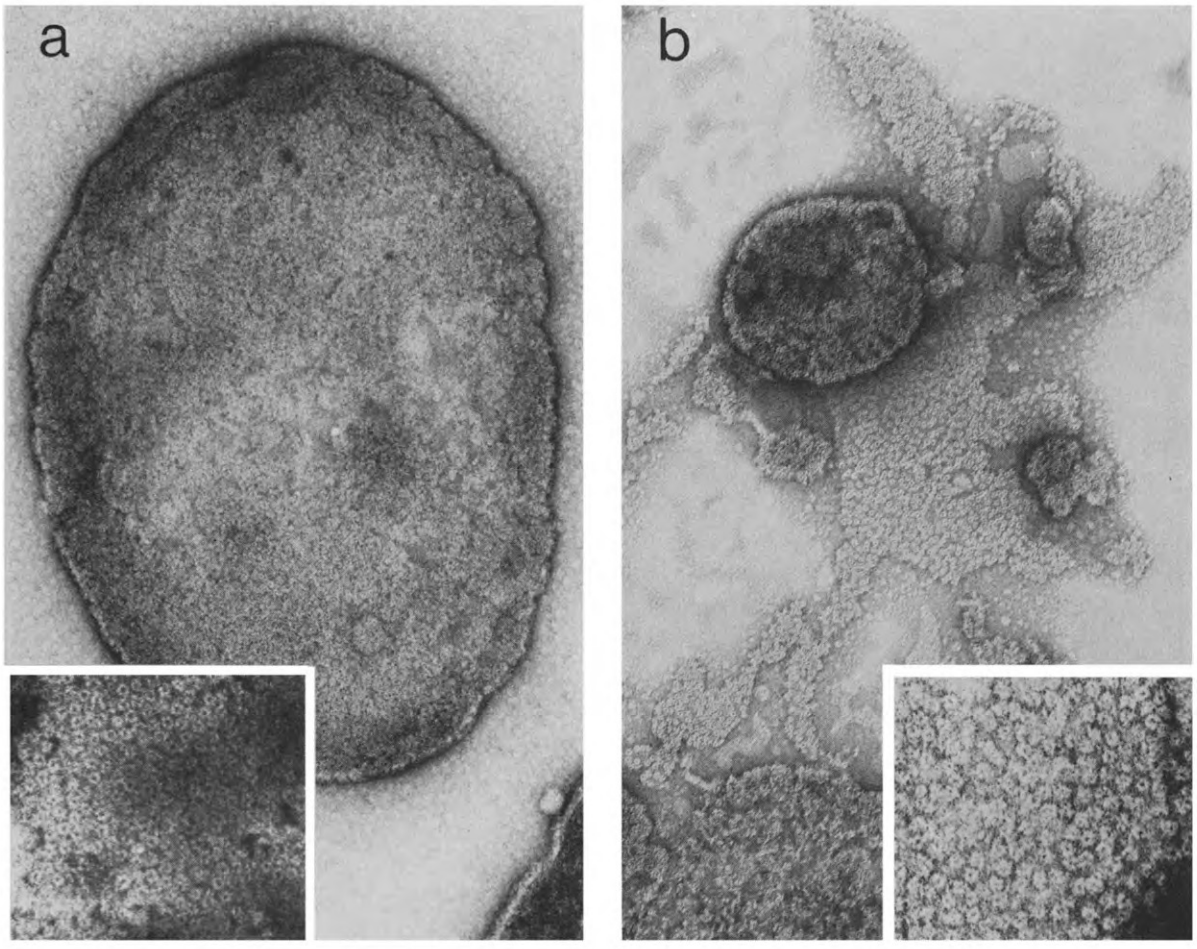


Fig.3. The effects of alkaline treatment on membrane structure as seen in negatively-stained microsacs from *T. marmorata*. (a) An untreated microsac (control). The typical rosette-shaped particles are densely packed, but areas relatively free of particles are also present. Therefore, the mean particle density determined by counting is lower than the theoretical maximum density for close packing. (b) After alkaline treatment a large proportion of membrane sheets, which are often fragmented, is observed. The particles in the membranes are wider spaced and more randomly distributed than in the control (see fig.4). The mean particle density, however, is comparable to that of the control. The extraction of peptides by high pH destabilizes the membrane, but without leading to its disintegration. The sheets are produced by surface forces during drying, which depend on the surface properties of the support film. The specimens of all micrographs are prepared on freshly made (hydrophilic) carbon films (see section 2): magn. 100 000 ×; insets 250 000 ×.

room temperature (fig.2a–c, 0 min). Heating of the samples at 100°C for increasing periods (2–5 min, fig.2), as is routinely done in many gel electrophoresis procedures under denaturing conditions, leads to a progressive loss of the ν-band. The sensitivity of the ν-band to heat treatment does not depend on the presence of high concentrations of reducing (100 mM dithiothreitol) or oxidizing (10 mM 5,5′-dithio-bis-(nitrobenzoic) acid) agents. As shown in fig.2, the progressive disappearance of the ν-band observed after 2 and 5 min boiling is not prevented by either of the extreme redox conditions employed in this study. This decrease in the intensity of the ν-band is accompanied by an increase in the low molecular weight components migrating in front of the M_r 40 000 band (fig.2).

3.3. *Differences between alkaline-treated membranes and control membranes*

There are three structural aspects:

(i) Upon drying onto EM grids coated with fresh carbon films, many membrane sheets as opposed to flattened microsacs are observed (fig.3). Many of these sheets correspond in size and shape to the control microsacs, although they often are fragmented. Of the original microsacs, 50–80% are affected in this way by the alkaline treatment; more precise values are difficult to obtain.

(ii) Compared to untreated microsacs, the typical AChR particles are more widely spaced. This applies not only to the sheets, but also to a sizeable proportion of the membrane vesicles found on old carbon films after alkaline treatment. In the control microsacs the spacings between nearest neighbour particles show a narrow, skewed distribution, peaked at 8.5 nm, with a mean of 9.7 nm (80% of the values are within 8–10 nm, fig.4). In contrast, the distribution of nearest neighbour spacings in alkaline treated membranes is rather broad, with an almost even spread of values between 8.5–13.0 nm (80% of the values). The mean distance is 11.7 nm, and 20% of the values fall between 14–18 nm (fig.4). The particle density, however, when averaged over sufficiently large membrane areas (0.15–0.20 μm^2) is, within the limits of error, the same in control membranes and in membrane sheets observed after the alkaline treatment (5000–6000/μm^2). This value is considerably less than the value (15 000/μm^2) that would be obtained either by a theoretical estimation based on ideal close packing (at interparticle spacings of 9.0 mm) or by counting particles exclusively within the densely packed areas

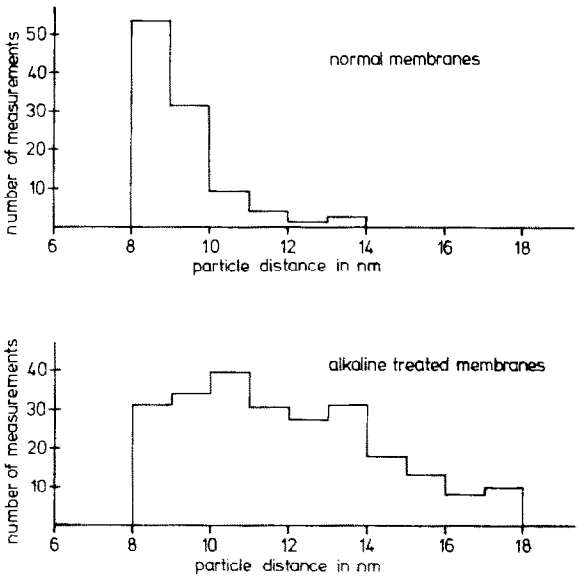


Fig.4. Distribution of nearest neighbour distances between the typical particles seen in negatively stained microsacs. Upper graph: Densely packed area from untreated microsacs. Lower graph: Membrane sheets after alkaline treatment. The histograms show that the alkaline treatment leads to an increase in the mean distance and to a randomization of the particle arrangement.

(9500/μm^2). It should be noted that generally microsacs tend to be inhomogeneous, i.e., densely-packed patches and patches relatively free of particles are often observed side by side within the same microsac membrane. From this follows that incubation at pH 11.0 produces a more even distribution of the particles over the entire membrane, resulting in an increase in the mean particle spacing.

Other conditions of pH (2.5–10.0), ionic strength, temperature or treatment with detergents or lysolecithins (below concentrations leading to complete solubilization) were not found to increase the interparticle spacing as assessed by electron microscopy.

The structural changes on the membrane level effected by the alkaline treatment were also observed in membranes which had not been washed after the incubation at pH 11.0. The changes could not be reversed in such samples by re-adjusting to pH 7.0. In all cases the preparations did not change in their morphology when stored at 4°C for several days.

(iii) Although the rosette shape of the particles is retained after alkaline treatment the particles appear somewhat less compact in comparison to those in

untreated membranes. A representative description, however, of the structure of individual AChR particles must await the application of image analysis and averaging [13,14]. Such a study may also help to clarify if the M_r 43 000 peptide is located within the doughnut-shaped particles.

4. Discussion

A procedure as simple as alkaline treatment of the AChR-rich membranes [9] results in considerable purification of the microsac fraction. Although the alkaline extraction does not show absolute selectivity for any of the peptides normally present in the AChR-rich membrane, the predominant component of the soluble extract is a band of M_r 43 000 detected by gel electrophoresis under denaturing conditions. This peptide seems therefore to be a loosely bound, normal component in the AChR-rich membrane from *Torpedo* electric organs. The M_r 43 000 band consists in fact of a doublet, here referred to as the ν-doublet. The sensitivity of the polypeptide pattern of the AChR-rich membrane (not subjected to alkaline treatment) to the conditions of solubilization, temperature and redox state may bear on the variability of the AChR subunit composition found in the literature [3].

Despite the substantial extraction of polypeptide components the membrane does not disintegrate, although the rigidity of the microsacs is noticeably reduced. Many membrane sheets are observed when alkaline treated microsacs are deposited on hydrophilic support films. These sheets are obviously produced by surface forces which act during drying [18], since very few sheets are found when such fragile membranes are put onto hydrophobic supports. The significant increase in interparticle spacing, at an unchanged overall particle density, is another striking manifestation of the membrane destabilization.

The preservation of the acetylcholine binding and ion transport properties in alkaline-treated membranes shown in [9,19], makes it unlikely that the M_r 43 000 peptide is necessary for these functions of the AChR protein. The structural changes observed after treatment with pH 11.0 seem to be specifically related to the peptide extraction. As mentioned in section 3, other procedures aimed at influencing the clustering of the receptor particles were unsuccessful. Therefore we consider it unlikely that the effects observed after alkaline treatment are merely secondary effects caused by pH 11.0 and unrelated to the loss of the ν-peptide.

Normally, the AChR in microsacs shows rotational immobilization on the time scale accessible to ESR measurements [15]. It has also been reported that there is relatively little lateral mobility of the AChR clustered in high density areas in developing muscle [16]. In qualitative agreement with this, we have observed a remarkable resistance of the particle arrangement towards various manipulations. The observation of changes in particle clustering after the alkaline extraction introduces a new structural aspect. These changes appear to be related to the removal of the M_r 43 000 peptide and are most plausibly explained by an increased freedom of motion of the AChR in the microsac membrane. Preliminary results from phosphorescence spectroscopy indeed show that the mobility of eosin–α-bungarotoxin-labelled receptor is increased after alkaline treatment (Bartholdi, F.J.B., Jovin, in preparation). Therefore we believe that the M_r 43 000 peptide may contribute to the clustering of the AChR particles in the membrane. It is tempting to speculate that some of the extracted peptides are involved in processes like synapse formation during development, the clustering of receptors, or the stabilization of adult synaptic patches (reviewed [4,17]).

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Note added

After submission of this manuscript, a report (Saitoh et al., FEBS Lett. (1979) 108, 489–494) was published, which shows that removal of the M_r 43 000 peptide drastically reduces the thermal stability of the receptor without affecting its functional integrity, as assessed by its ability to bind α-bungarotoxin.

We have shown that membrane fragility and particle clustering are altered by pH 11, but not by pH 10. This range encompasses the range (pH 10.4–10.8), for which maximal peptide extraction, the largest change in heat stability, but negligible loss of toxin binding activity occurs (Saitoh et al., op. cit.). In order to reduce the binding capacity by alkaline treatment pH values >11 are required (Saitoh et al., op.

cit.). By combining the results of the two studies, it appears likely that the M_r 43 000 polypeptide awards thermal and motional stability to the receptor protein.

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