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STRUCTURAL CHANGES IN ALKALINE-TREATED POSTSYNAPTIC MEMBRANES FROM *TORPEDO MARMORATA* ARE NOT DUE TO LIPID HYDROLYSIS

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The removal of the 43 kDa peptide from postsynaptic membranes from the electric organ of *Torpedo* by alkaline treatment (pH 11) or by lithium diiodosalicylate at pH 8 results in similar ultrastructural changes that cannot be produced by the action of porcine pancreatic phospholipase A_2 or lysophosphatidylcholine. Thin-layer chromatography fails to reveal significant alkaline hydrolysis of membrane lipids from postsynaptic membranes and erythrocyte ghosts under the conditions used for peptide extraction.

Recently, the purification of the acetylcholine receptor protein in its membrane-bound form from the electric organ of *Torpedo* has been improved by introducing an alkaline washing step. This results in the removal of certain membrane-associated peptides, notably of a peptide with a molecular weight of 43000 [1] (see also Fig. 1). It is now agreed that this peptide is not involved in the function of the receptor nor in the binding of local anaesthetics [2]. However, the stability of the membrane is reduced and the mobility of the acetylcholine receptor protein is increased [3–6]. The observed changes were ascribed to the removal of the 43 kDa peptide. The question arose whether these changes could be due to alkaline hydrolysis of membrane lipids. For erythrocyte membranes this possibility has already been discussed and considered unlikely by Steck and Yu [7].

Our experiments, primarily aimed at resolving this point for the postsynaptic membranes, answer the question also for human erythrocytes.

In order to save material, all experiments connected with lipid analysis were first carried out on erythrocyte ghosts, which were prepared according to Dodge et al. [8] from human outdated blood, so that only the decisive tests had to be done on *Torpedo* membranes.

The acetylcholine receptor-rich membrane fraction (microsacs) from *Torpedo marmorata* was purified and assays of its protein content and specific activity were carried out as described by Barrantes et al. [4,9].

The stock suspension contained 37% sucrose, 0.02% NaN_3, 1 mM EDTA and membranes with a protein concentration of 10 mg/ml. Their specific activity ranged from 1.7 to 2.2 nmol α-bungarotoxin bound per mg of protein. All observations were made on at least two different preparations. The stock suspension was diluted to 0.5 mg/ml protein with a solution of about 1 mM or about 10 mM NaOH (pH 11 or 12). Final volumes were 50 μl, and were stirred for 1 h at room temperature or at 80°C with a self-made flea in a 0.5-ml Eppendorf reaction vessel. The suspension was centrifuged in a Beckman Airfuge (20 psi, 30 s) and the pellet resuspended in distilled water.

Treatment with NaOH at pH 11 releases loosely associated peptides from the membranes [1]. The

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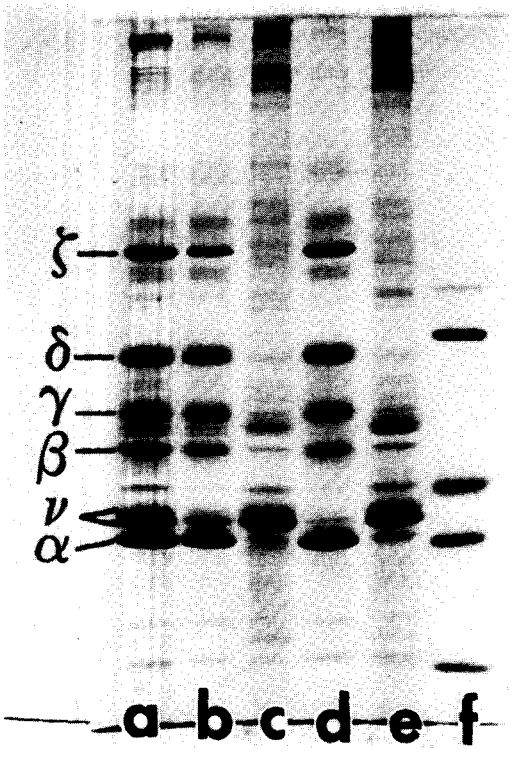

Fig. 1. SDS-polyacrylamide patterns of microsacs in a Laemmli system [19] (stacking gel of 5% and separating gel of 10%). 20 μg of protein were applied per track as described by Barrantes et al. [4]. (a) Polypeptide composition of the acetylcholine receptor membranes used as the starting material. The gel pattern shows the four acetylcholine receptor subunits (α, β, γ, δ) and in addition, the non-receptor peptide of $M_r = 43000$ (also called ν-protein [4]). Additionally, *Torpedo* actin [20] and the heavy chain of the ATPase (ζ) [21] are usually present in the membrane fraction. (b) Same as (a) after 1 h of NaOH treatment (20°C, pH 11). (c) Supernatant recovered from a 19750 × g centrifugation of (b). (d) Membranes depleted of the 43 kDa peptide by extraction with lithium diiodosalicylate (10 mM, pH 8.0) show the characteristic bands (α, β, γ, δ) of the acetylcholine receptor subunits. (e) Supernatant of (d), which is similar to that after alkaline treatment. (f) From top to bottom: bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), aldolase (M_r 40000), and chymotrypsin (M_r 26000).

extraction of the 43 kDa peptide under these conditions is not quite complete (Fig. 1b) but except for a minor depletion of the β-subunit the receptor bands are not affected. As shown in Fig. 1c, the most conspicuous component released by the alkaline treatment is the 43 kDa peptide.

Electron microscopic specimens were prepared from membrane suspensions containing 0.5–1 mg/ml protein. 2 μl of the suspension were put onto carbon-coated copper grids. The carbon films had been washed with a 0.1% solution of poly-lysine and dried before use. The washing with poly-lysine produces a uniform and reproducible hydrophilic surface [4]. The specimens were washed by floating on three droplets of water for 5 min each time and stained with uranyl formate. Micrographs were taken with a Phillips EM 301 at 80 kV. The diameter of the molecules was measured on micrographs at a final magnification of 375000 ×.

Our results concerning the morphology of alkaline-treated acetylcholine receptor-rich membranes clearly show that membranes from which the 43 kDa peptide is largely removed can be distinguished visually from those that still contain the peptide. The difference expresses itself on three different levels. (1) The tendency to form membrane sheets [4]. (2) Distribution of receptor molecules within the membrane [4]. (3) The structure of the receptor complex and its immediate environment.

The first property may easily escape observation since the extent of sheet formation is strongly influenced by the interaction of surface forces between the membranes and the support [4,10]. The tendency to form sheets varies between different preparations, but this tendency is always increased by alkaline treatment [4].

The distribution of receptor molecules within the membrane is always more random and less dense after the removal of the 43 kDa peptide [4]. This is evident in sheets as well as in vesicles and can also be noted in micrographs published by other groups [11,12].

Changes of the receptor complex itself are obvious after image analysis (manuscript in preparation, see also Ref. 13). However, one altered feature is detectable by conventional methods. After the removal of the 43 kDa peptide by alkaline pH the typical rosettes seen in negatively stained specimens are larger (see Fig. 2). The increase in size is about 10% of the molecular diameter. This agrees well with the results of image analysis [13].

An alternative method for removing the 43 kDa peptide from acetylcholine receptor-rich membranes is the incubation with lithium diidosalicy-

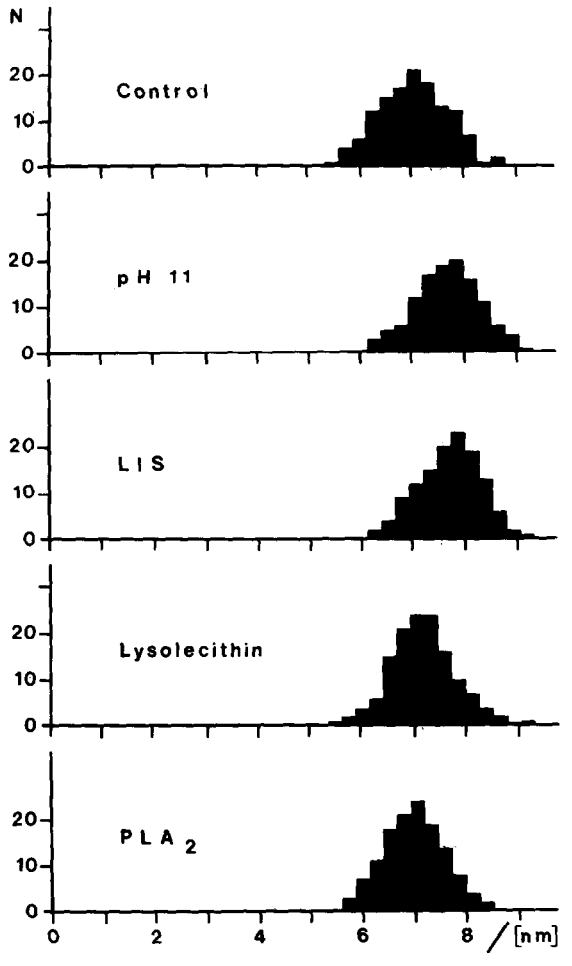

Fig. 2. Histograms showing the diameter of acetylcholine receptor molecules after various treatments (in nm vs. the number of molecules) as determined by electron microscopy of negatively stained specimens. The mean diameter (d) and the standard error of the mean (S.E.) were determined and the respective values are given below ($d \pm$ S.E.). The diameter of membrane-bound acetylcholine receptor molecules (7.0±0.07 nm) is increased reproducibly by about 10% after treatment with pH 11 (7.8±0.07 nm) or with lithium diiodosalicylate (LIS) (7.7±0.08 nm). Treatment with lysolecithin (7.2±0.06 nm) or with phospholipase A_2 (PLA_2) (7.0±0.06 nm) has no such effect. From these values it is clear that the 10% change after removal of the 43 kDa peptide is significant. The lithium diiodosalicylate concentration was 10 mM, the concentration of lysophosphatidylcholine was 10% of the total lipid.

late [12]. Membranes in 10 mM sodium phosphate buffer (pH 8.0) at a concentration of 1 mg/ml protein were incubated with various concentrations of lithium diiodosalicylate (Sigma) for 30 min on ice. This treatment accomplishes an even more complete extraction of non-receptor peptides than incubation at pH 11 (Fig. 1d). However, this agent disintegrates membranes [14,7]. Elliott et al. [12] used 20 mM lithium diiodosalicylate to remove the 43 kDa peptide, but already at 5 mM the vesicles are severely damaged. They form large aggregates in which the shape of the vesicles cannot be discerned. Areas amenable to electron microscopic evaluation are rare in such preparations, but can be found if concentrations of lithium diiodosalicylate not exceeding 10 mM are used. The size and distribution of acetylcholine receptor molecules treated with lithium diiodosalicylate is indeed very similar to those of alkaline-treated membranes (see Fig. 2).

In order to observe the effect of lysophosphatides on the membrane morphology the microsacs at a concentration of 1 mg/ml protein (corresponding to 0.5 mg/ml lipid, see Ref. 12) were incubated with different amounts (5–20% of total lipid) of lysophosphatidylcholine (1-palmitoyl-*sn*-glycero-3-phosphocholine, a gift from Dr. H. Eibl) for 30 min at room temperature. For the same purpose the membranes were also treated with phospholipase A_2. The microsacs in 50 mM Tris-HCl (pH 7.4) and 2.5 mM $CaCl_2$ were incubated with 1 I.U. phospholipase A_2 from porcine pancreas (Sigma) for 5 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 5 mM.

The treatment of the membranes with phospholipase A_2 or lysophosphatidylcholine produces changes of vesicle shape, but does not induce sheet formation or any other of the effects of alkaline treatment or incubation with lithium diiodosalicylate. In particular, no increase in molecular diameter is observed (Fig. 2). Thus, none of the effects of alkaline treatment can be mimicked by manipulating the lipid composition of postsynaptic membranes.

The conclusion that the observed changes are indeed due to the removal of the 43 kDa peptide and unrelated to lipid hydrolysis is strenthened by the results of lipid thin layer chromatography. Lipids were extracted as described by Renkonen et al. [15]. The chromatograms in one or two dimensions were developed according to Broekhuyse [16] on Merck silica gel 60 in chloroform/

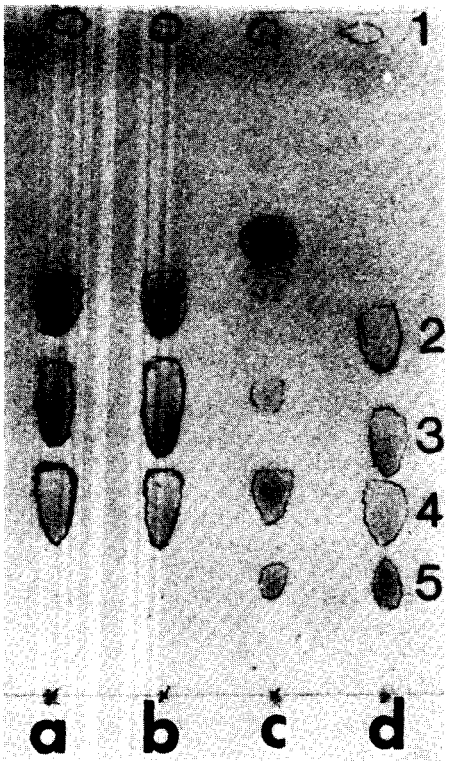

Fig. 3. Thin-layer chromatogram of acetylcholine receptor-rich membrane lipids in $CHCl_3/CH_3OH/NH_3/H_2O$ (90:54:5.5:5.5, v/v). About 100 μg of lipid were applied to each track. (a) Lipids from untreated acetylcholine receptor-rich membranes. (b) Lipids from acetylcholine receptor-rich membranes after 1 h incubation at pH 11 at room temperature. (c) Lipids from acetylcholine receptor-rich membranes after 5 min incubation with phospholipase A_2 at 37°C. (d) Synthetic lipids: cholesterol (1), phosphatidylethanolamine (2), phosphatidylcholine (3), phosphatidylserine (4), lysophosphatidylcholine (5). The spots were immediately marked with pencil because the iodine stain fades rapidly. The faint spots in track (c) stand out clearly in the original chromatogram when observed through a blue filter. Between the patterns of track (a) and (b) there is no difference. In particular, no traces of lysophosphatides are detectable even through a blue filter. Under our condition 1 μg of lysophosphatidylcholine would have been detectable. Thus, if hydrolysis occurs at all, the proportion of lysophosphatides is less than 1% of total lipids (corresponding to less than 2.5% of phosphatidylcholine [17]).

methanol/conc. ammonia/water (90:54:5.5:5.5, v/v) in the first dimension and in chloroform/methanol/acetic acid/water (90:40:12:2, v/v) in the second dimension (see Ref. 17). The chromatograms were stained with iodine vapour.

The limit of detection of lysophosphatidylcholine in iodine stained chromatograms was 1 μg. Using 100 μg of lipid in each experiment, the chromatograms of lipids from acetylcholine receptor-rich membranes treated with pH 11 and from untreated membranes were identical (Fig. 3a and b). The same was true for lipids from erythrocytes, even if 20-fold more lipid was used. Additional spots appeared in samples which were treated with phospholipase A_2 (Fig. 3c). Incubation of erythrocyte ghosts at pH 12 did not produce lysoderivatives except when the temperature was 80°C during the 1 h incubation (data not shown). In contrast, pure phosphatidylcholine (2 mg in 50 μl ethanol, injected into 3 ml buffer and treated as the membranes) is hydrolysed to a considerable extent at pH 11 (data not shown). However, other lipids are much less sensitive to alkaline hydrolysis and may even protect phosphatidylcholine when mixed with it (Eibl, H., personal communication). Therefore, it would not even be necessary to invoke a protecting effect by the membrane protein.

In conclusion there are three lines of evidence that lipid hydrolysis is negligible under the conditions for alkaline extraction of the 43 kDa peptide:

(1) The chromatograms of control and alkaline-treated membrane lipids are identical. About 40% of the lipids in *Torpedo* acetylcholine receptor-rich membranes are phosphatidylcholines [17]. Under our experimental conditions hydrolysis of less than 2.5% of total phosphatidylcholine would have been detected in the chromatograms. The virtual absence of lysoderivatives is also consistent with the results of Rousselet et al. [5]. There was no support for the suspicion that the increased mobility of the acetylcholine receptor molecules after alkaline treatment could be due to lysophosphatidylcholine, because washing with albumin (which is expected to extract lysophosphatidylcholine [18]) did not decrease the mobility.

(2) Incubation of membranes with sublytic concentrations (up to 20% of total lipid) of lysophosphatidylcholine does not produce effects comparable to those observed after alkaline treatment.

(3) Lithium diiodosalicylate, which removes the 43 kDa peptide, but is not expected to produce lysophosphatidylcholine, induces similar ultrastructural changes as alkaline treatment (see also Fig. 2).

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