Consequences of Alkaline Treatment for the Ultrastructure of the Acetylcholine-receptor-rich Membranes from *Torpedo marmorata* Electric Organ

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ABSTRACT   After fixation with glutaraldehyde and impregnation with tannic acid, the membrane that underlies the nerve terminals in *Torpedo marmorata* electroplaque presents a typical asymmetric triple-layered structure with an unusual thickness; in addition, it is coated with electron-dense material on its inner, cytoplasmic face. Filamentous structures are frequently found attached to these "subsynaptic densities." The organization of the subsynaptic membrane is partly preserved after homogenization of the electric organ and purification of acetylcholine-receptor (AchR)–rich membrane fragments. In vitro treatment at pH 11 and 4°C of these AchR-rich membranes releases an extrinsic protein of 43,000 mol wt and at the same time causes the complete disappearance of the cytoplasmic condensations. Freeze-etching of native membrane fragments discloses remnants of the ribbonlike organization of the AchR rosettes. This organization disappears after alkaline treatment and is replaced by a network which is not observed after rapid freezing and, therefore, most likely results from the lateral redistribution of the AchR rosettes during condition of slow freezing. A dispersion of the AchR rosettes in the plane of the membrane also occurs after fusion of the pH 11-treated fragments with phospholipid vesicles. These results are interpreted in terms of a structural stabilization and immobilization of the AchR by the $43,000$-M_r protein binding to the inner face of the subsynaptic membrane.

In the electroplaque (10) and at the neuromuscular junction (22), the local distribution of the acetylcholine receptor (AchR) in the subsynaptic membrane determined by high resolution autoradiography does not change for days or even weeks after denervation. Fluorescence photobleaching experiments disclose an almost complete absence of lateral mobility of the α-toxin-labeled AchR in subsynaptic areas of the adult neuromuscular junction (4, 19). Finally, measurements of the rotational motion of the spin labeled AchR by electron spin resonance (ESR) in AchR-rich membranes from *Torpedo marmorata* reveal a strong immobilization of the receptor molecules within a relatively fluid lipidic environment (8, 46), suggesting that protein-protein interactions may account for the remarkable structural stability of the subsynaptic membrane.

The receptor-rich membranes prepared from *T. marmorata*, therefore, represent an excellent model system to investigate the molecular mechanisms responsible for the localization and stabilization of the AchR in the subsynaptic membrane. In addition, they give a rather simple pattern of polypeptides after one-dimension polyacrylamide gel electrophoresis (PAGE) in SDS (reviewed in references 16, 30, 42): two major bands of apparent mol wt 40,000 and 43,000 and a few minor ones of apparent mol wt 50,000, 60,000, and 66,000. The 40,000 mol wt chain unambiguously carries the acetylcholine binding site (31), and the 66,000 mol wt chain is labeled by a photoaffinity derivative of trimethisoquin (41, 48), a noncompetitive blocker of the permeability response to acetylcholine. Neubig et al. (39) have shown that in vitro alkaline treatment of the AchR-rich

membranes causes the release of the 43,000 mol wt polypeptide without significantly altering the known functional (binding and ion transport) properties of the AchR-rich membranes (see also references 27, 52).

The experiments we report are designed to shed some light upon the functional role of the 43,000 mol wt protein (6, 53) in the receptor-rich membrane form *T. marmorata*. The ultra-structural data presented here show that, upon removal of the 43,000 protein by alkaline extraction, a dense material adherent to the inner face of the membrane disappears and a destabilization of the membrane occurs. These results are consistent with spectroscopic data which indicate that, under the same conditions, a marked increase of the rotational motion of the receptor molecules occurs (36, 45). Some of these results have been briefly reported (52).

MATERIALS AND METHODS

Preparation of AchR-rich Membranes from T. Marmorata

The membrane fragments were purified from fresh electric tissue, following the procedure (52) modified from Sobel et al. (54). A second sucrose gradient centrifugation was always performed. Finally, the membrane were resuspended in distilled water or modified Torpedo Buffer (250 mM NaCl, 5 mM KCl 10 mM Tris, pH 7.2). When specified, proteolysis was inhibited during purification by a mixture of antiproteolytic agents as described in (48).

Alkaline Extraction of the 43,000 Mol Wt Protein

The purified membranes were treated at pH 11 by a procedure derived from the method of Neubig et al. (39): alkaline extraction was carried out as described in Sobel et al. (52) but at low temperature (4°C for 60 min).

Most of the electron microscopic experiments we describe were done in parallel with electron spin resonance experiments in which a reduction step was needed for the labeling (see reference 45, 46); a number of alkaline-treated samples presented here were also reduced. No significant differences in the electron microscopic appearance were apparent between reduced (either 2 mM DTT or 20 mM β-mercaptoethanol) and nonreduced membranes.

PAGE

PAGE of native and alkaline-treated membranes was performed in the presence of SDS on 10% acrylamide slab gels described in Sobel et al. (52) by the method of Laemmli (34) modified by Anderson and Gesteland (3). Fig. 1 *a* shows the pattern of polypeptide chains given by one-dimensional SDS PAGE of *T. marmorata* AchR-rich membranes purified in the presence of EDTA to prevent proteolysis, after staining by Coomassie Brilliant Blue. Protein bands with apparent molecular weights of 40,000, 43,000, 50,000, 60,000, 66,000 and 95,000 daltons (determined with water-soluble proteins as standards) were observed in addition with other minor components. After alkaline extraction, the 43,000 and 95,000 bands disappeared (Fig. 1 *b*). The four remaining polypeptides correspond to the α, β, γ, and δ chains of the AchR (16, 30). Membranes prepared following the conventional procedure in the absence of EDTA (52) gave patterns characterized by a decrease in the intensity of the β, γ, and δ bands due to proteolysis and by an almost complete absence of 95,000 mol wt component. Compared electron microscopic observations of negatively stained samples of both types of preparation showed that contaminating membrane fragments, probably derived from the noninnervated ATPase-rich face of the electroplaques, were present in quantities roughly proportional to the amount of the 95,000 mol wt component.

Modification of the Membrane Lipid Phase

Addition of phospholipids (phosphatidylcholine) to native and alkaline-treated membrane fragments was done by fusion with sonicated phospholipid vesicles. Typically, 2 ml of a 3 mM phosphatidylcholine suspension was mixed with 2 mg of membrane proteins. After incubation for 12 h at 10°C under nitrogen, the membrane suspension was washed three times with buffer to eliminate the lipid vesicles absorbed at the membrane surface. Attempts to separate the fused membranes from the nonfused ones by sucrose gradient centrifugation were unsuccessful. We selected in the microscope the fragments showing obvious AchR-free areas and/or fragments with scattered rosettes, which accounted for 20–50% of the total.

Electron Microscopy

SECTIONS, FIXATION, AND EMBEDDING: Aliquots of membrane suspension were fixed in 2.5% glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, England; E.M. grade) in 0.1 M sodium cacodylate buffer (pH 7.2) containing 1% (wt/vol) tannic acid (Mallinckrodt analytical reagents; 38, 47, 51, 55) for 2 h at 4°C. Fixed membranes were centrifuged (30 min, 30,000 rpm in rotor Beckman SW50L, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), washed, and finally postfixed in 1% OsO₄.

Small blocks of freshly dissected electric tissue were processed for tannic acid impregnation according to Simionescu and Simionescu (55). Various sequences of impregnation were tested, and the following sequence was finally adopted: glutaraldehyde (2.5% in 0.2 M cacodylate buffer), tannic acid (1% in the same buffer), and OsO₄ (1% in 0.1 M cacodylate buffer).

All samples were dehydrated through a graded series of acetones or alcohols and embedded in Vestopal (membrane fragment) or Epon (blocks of tissue). Thin sections were stained with 1% uranyl acetate in 50% ethanol for 10 min and counterstained with lead citrate (43).

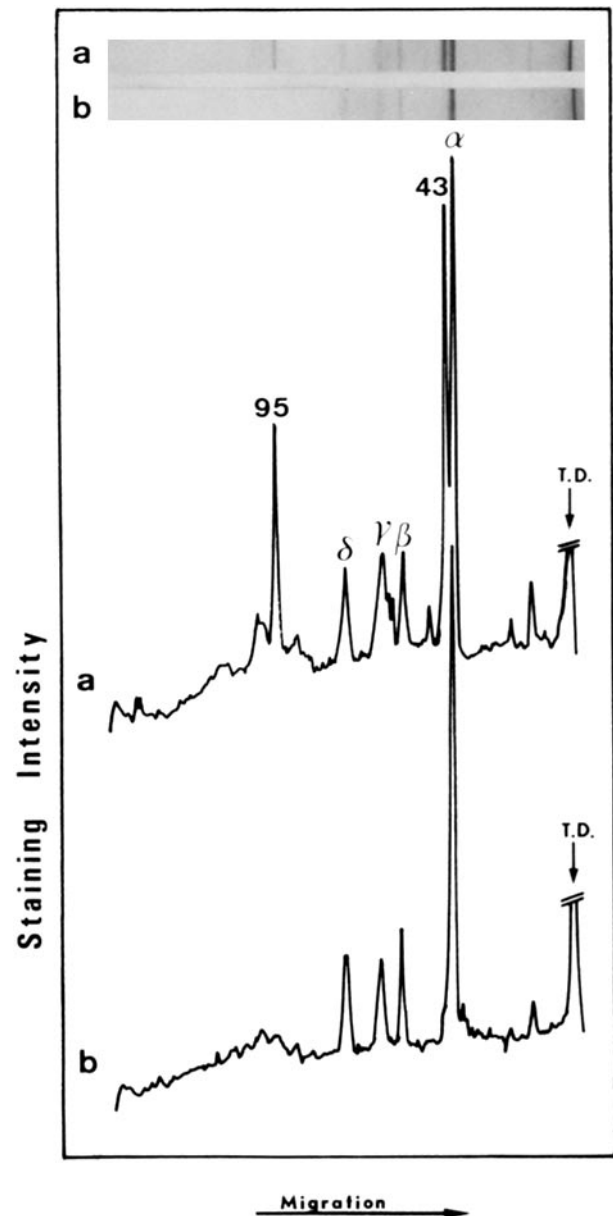NEGATIVE STAINING: All samples were diluted before use in 13 mM ammo-

FIGURE 1   SDS PAGE of (*a*) native (~15 μg of protein) and (*b*) alkaline-extracted (~10 μg protein) *T. marmorata* membrane fractions prepared in the presence of antiproteolytic agents. Top: Coomassie Brilliant Blue staining. Bottom: densitometric scan of the gels shown on top. T. D.: tracking dye.

nium formate to a final concentration of ~0.5 g protein/ml. The negative staining procedure was almost similar to that already described (14). Thin carbon films supported on fenestrated plastic-carbon films were prepared according to Fukami and Adashi (23). Uranyl formate was prepared as described by Leberman (35) and used as a 1% aqueous solution.

FREEZE ETCHING: Purified membrane fragments were frozen directly without chemical fixation or glycerination. For conventional freeze-etching experiments, the concentrated membrane suspension (15–20 mg protein/ml) was deposited as small drops on Balzers gold discs (Balzers Corp., Nashua, N. H.) and frozen, from room temperature, in liquid-solid Freon R-22 cooled in liquid nitrogen. For rapid freezing, the device developed by Dr. T. Gulik-Krzywicki was used (25). A very small drop of the concentrated suspension (0.1 μl) was compressed between two thin copper plates separated by a single-holed (1 × 1.5 mm) electron microscope grid. After freezing in Freon R-22 at −160°C, the specimen was mounted on a specially designed table in the Balzers machine and then manually split. Fracturing was achieved by a few cuts of the cold microtome arm fitted with the usual ultrasharp razor blade, and both types of specimens were etched for 2–3 min at −100°C under vacuum >10⁻⁶ torr and then replicated. Replicas were cleaned in sodium hypochloride and mounted on hexagonal 400-mesh grids.

OBSERVATION IN THE ELECTRON MICROSCOPE: All the 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. The observations of negatively stained specimens were made with a low emission current (typically 10 μA), and pictures were taken without pre-exposure of the specimen to the electron beam.

RESULTS

Ultrastructure of the Subsynaptic Membrane In Situ after Tannic Acid Impregnation

Fragments of fresh electric organ from *T. marmorata* were fixed with glutaraldehyde and impregnated with tannic acid following the method of Simionescu and Simionescu (55; see Materials and Methods). Fig. 2 confirms (see references 11, 44, 51) that the subsynaptic membrane presents an asymmetric triple-layered structure but an unusual thickness for a cytoplasmic membrane. The outer lamina (~7 nm thick) often

disclosed a 9- to 10-nm periodicity interpreted as representing the externally exposed moiety of the AchR molecules (11, 44, 51). On its cytoplasmic face, the inner lamina appeared coated by a dense material or "condensations" except over short segments where the postsynaptic membrane became very thin and its asymmetric structure disappeared.

A number of irregular filaments, whose nature is under investigation, straddled the cytoplasm of the electroplaque that underlay the subsynaptic membrane. These filaments were frequently found with end-on attachment to the cytoplasmic condensations (Fig. 2c; see also references 28 and 44).

Isolated AchR-rich Membrane Fragments

After glutaraldehyde fixation and tannic acid impregnation, the ultrastructure of the highly purified AchR-rich membranes (see Materials and Methods) resembled that of the subsynaptic membrane *in situ*, with its asymmetric triple-layered organization and cytoplasmic condensations (Fig. 3). It appeared remarkably thick (~11 nm), with a 6- to 7-nm thick outer dense layer (Fig. 3b) displaying a 9-nm linear periodicity (Fig. 3b and d). At the cytoplasmic face of the membrane fragments, remnants of the condensations made irregularly distributed patches (Fig. 3c and d). Filaments were no longer found attached to the cytoplasmic condensations. On the other hand, many of them were trapped within a small fraction of vesicles (which probably was derived from the dorsal, noninnervated face of the electroplaques) and contaminated the preparation (data not shown).

As already described, negatively stained preparations of purified membranes showed the characteristic AchR rosettes, usually irregularly packed in the plane of the membrane sheets (see references 1, 5, 12, 13, 40, 58). Freeze-etching experiments,
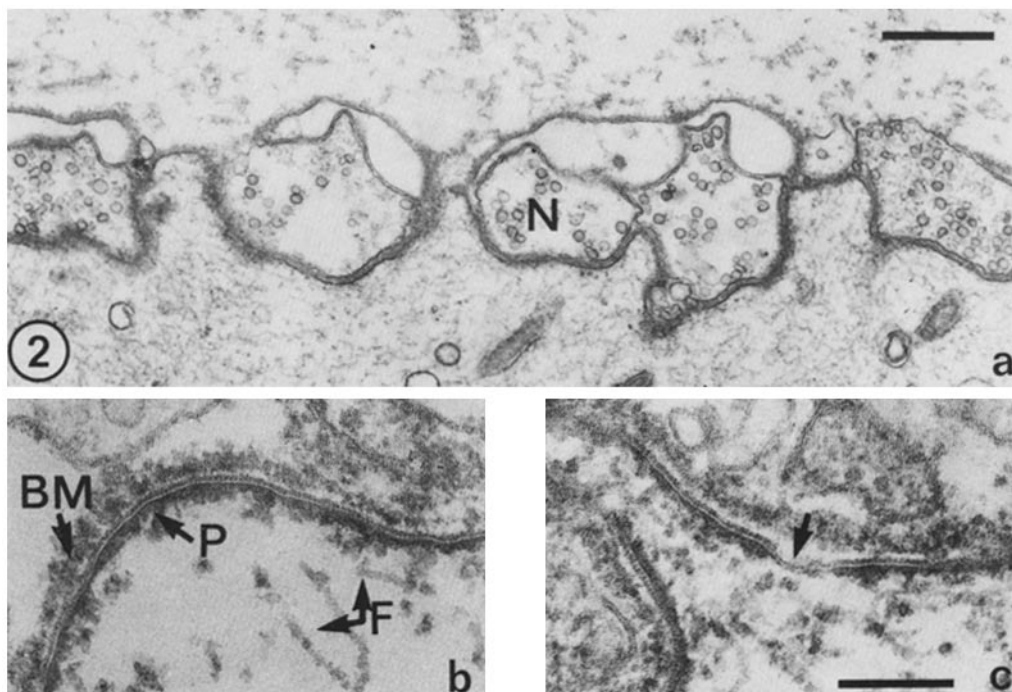
FIGURE 2   *T. marmorata* electroplaque after tannic acid–glutaraldehyde fixation. (a) Survey view of the innervated (ventral) face of the cell with numerous nerve endings (N) × 15,000. (b and c) Detailed views of the subsynaptic regions. The asymmetric trilaminar appearance of the membrane and the dense amorphous coating adherent to its cytoplasmic face are evident. Note the irregular distribution of this dense material and its strict cospatial distribution with membrane differentiated domains (arrow in c). Numerous contacts between cytoplasmic filaments and the dense coat are visible (c). BM = basal membrane; F = filaments; and P = postsynaptic membrane. × 80,000 Bars: 1 μm in a and 0.2 μm in b and c.
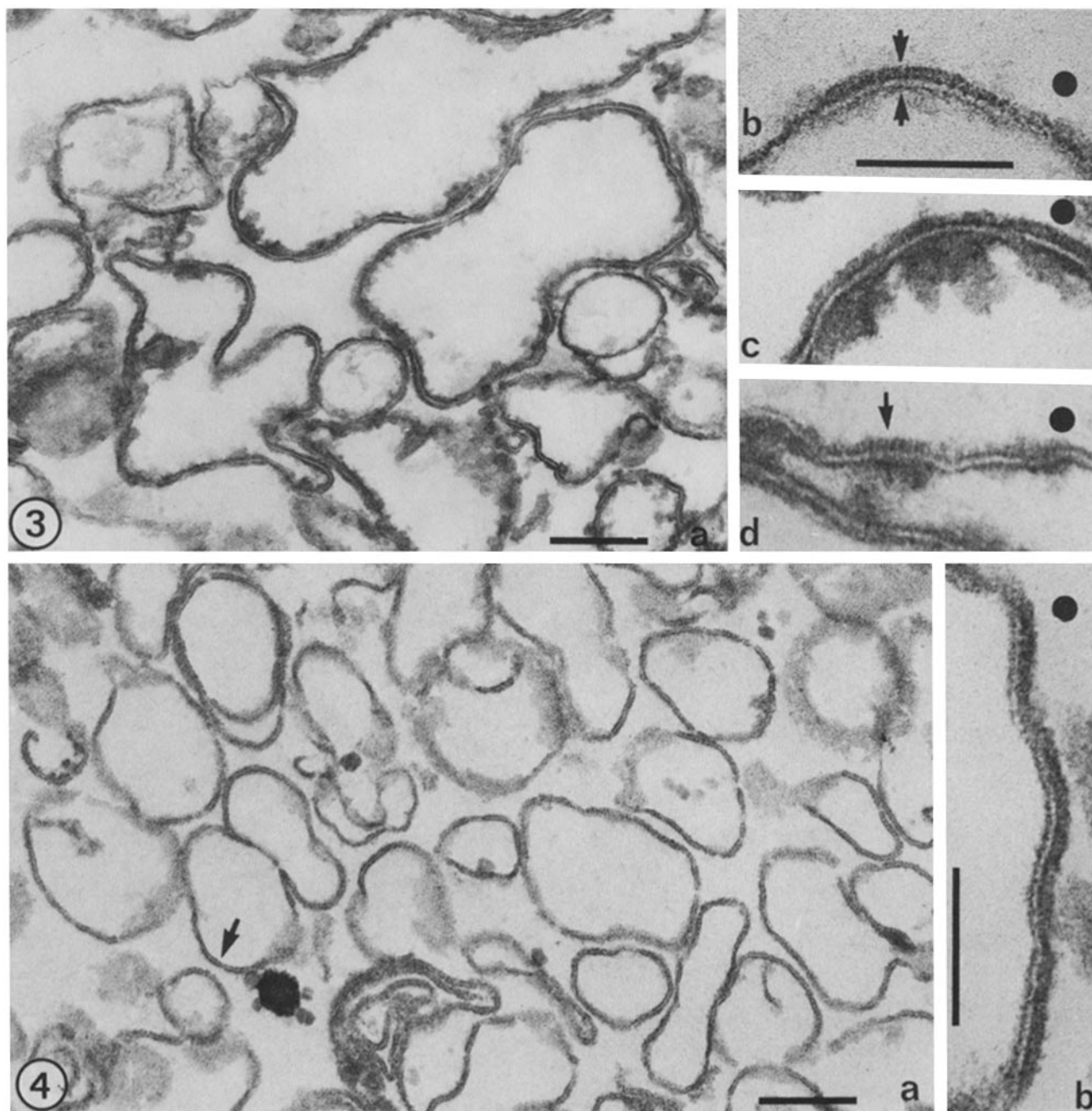
FIGURE 3   Isolated AchR-rich vesicles observed in thin section after tannic acid-glutaraldehyde-OsO₄ fixation. (a) Low magnification field showing several closed vesicles in which condensations of dense material adherent to the inner face of the membrane are visible. × 80,000. (b) Detail of a membrane showing the asymmetry of the membrane element, the widths of the membrane trilayer being from the outside (●) to the inside: 6:2.5:2.5 nm. A longitudinal periodicity is detectable in the outer dense layer. Few cytoplasmic condensations are present in this area. (c) Detail of a membrane fragment displaying cytoplasmic condensations. (d) Detail of a membrane fragment in which a clear cospatial localization of the outwardly exposed dense lamina and cytoplasmic coating material is exhibited (arrow). (b), (c), and (d): × 250,000. Bars: 0.2 μm in a and 0.1 μm in b, c, and d.

FIGURE 4   Alkaline-treated *T. marmorata* AchR-rich membrane fragments observed in thin section (tannic acid–glutaraldehyde-OsO₄ fixation). (a) The low magnification field reveals that the vesicular aspect of the fragments is essentially preserved, whereas the cytoplasmic coating material has completely disappeared. × 80,000. (b) Details of a membrane element which show the preservation of the asymmetric triple-layered structure (compare with Fig. 3 b). × 250,000. Bars: 0.2 μm in a and 0.1 μm in b.

including rapid freezing, carried out with these membranes, disclosed that the receptor rosettes bud on the outer surface of the vesicles. A ribbonlike organization consisting of linear arrays of paired receptor rosettes (which presumably correspond to the heavy form of the receptor; 15) was systematically but infrequently observed (Fig. 6; 13). Such an organization, however, had been consistently observed *in situ* (11, 28). Minor structural alterations may thus accompany the isolation and purification of the AchR-rich membranes. On the other hand, no significant structural differences were noticed by freeze-

fracture analysis between membrane fragments prepared in the absence (Fig. 6a and b) and in the presence of EDTA to prevent proteolysis (Fig. 6c).

Alkaline-treated AchR-rich Membrane Fragments

In agreement with the results of Neubig et al. (39), a quantitative release of the 43,000 mol wt polypeptide took place after pH 11 treatment (see Fig. 1); as a consequence, important modifications of the ultrastructure of the AchR-rich membranes were noticed. After tannic-acid glutaraldehyde fixation, the membrane fragments conserved their vesicular organization (Fig. 4a) and their transverse asymmetric triple-layered appearance (Fig. 4b), but the cytoplasmic condensations completely disappeared. The negatively stained preparations displayed the receptor rosettes with the same shape, size, distribution, and surface density (12,000 ± 4,000 per μm^2) as in the native membranes (Fig. 5).[1] After freeze-etching by the con-

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[1] Recent reports indicate that structural modifications of AchR-rich membranes observed by negative staining (6, 45, 52) after pH 11 treatment result from the fact that the alkaline treatments were performed at 20°C instead of 4°C as in our study. High temperature incubation at pH 11 causes drastic structural alterations of the membrane, accompanied, for example, by a loss of excitability (39) and lipid modifications.

FIGURE 5   Alkaline-treated AchR-rich membranes observed after negative staining. The vesicles display an almost normal structure compared to native fragments. Bar, 0.1 μm. × 200,000.
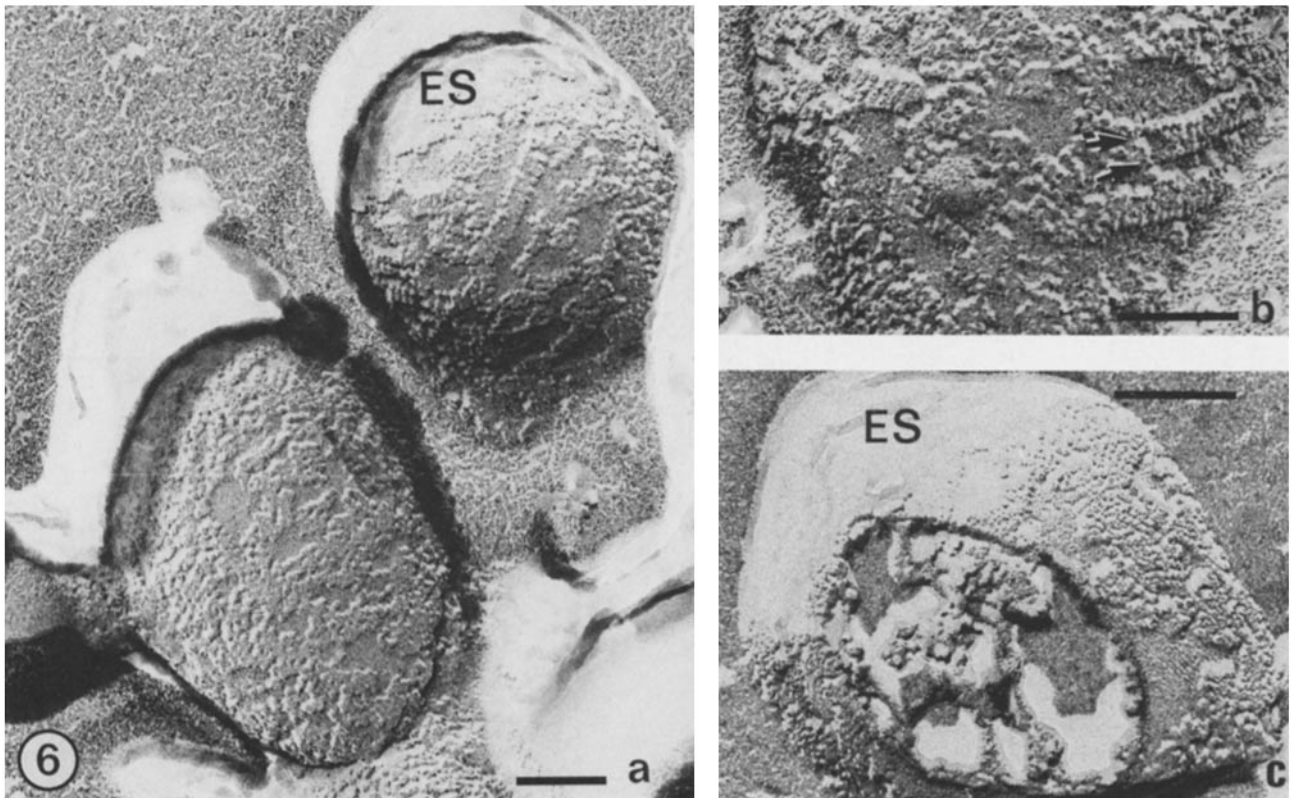

FIGURE 6   Freeze-etched aspect of native AchR-rich membrane fragments. (a) Conventionally freeze-etched membrane vesicles showing the typical distribution of the rosettes on ES. × 120,000. (b) Selected membrane area displaying remnants of double rows of paired rosettes. (c) Rapidly frozen sample (see Materials and Methods) in which no obvious modification in the distribution of the rosettes is observed. × 160,000. Bars, 0.1 μM.

ventional method (see Materials and Methods), a remarkable redistribution of the receptor rosettes took place. Instead of the closely packed appearance observed with the native membranes, the receptor rosettes made some kind of meshwork (Fig. 7 a and b) interspaced with smooth membrane areas. This redistribution affected nearly all the rosettes present on a given vesicle and was noticed with all the membrane fragments containing the receptor particles. Subsequent reduction of the membrane by 2 mM dithiothreitol/20 mM β-mercaptoethanol after pH 11 treatment did not modify the redistribution of the rosettes in conventionally frozen samples (Fig. 7 a and b). On the other hand, when the alkaline-treated membranes were examined after rapid freezing, little if any redistribution of the rosette was noticed (Fig. 7 c–e), indicating that this reorganization took place during slow cooling, probably as a result of

a segregation of protein and lipid domains of the destabilized membrane. The particles revealed by freeze-fracture on the inner leaflet (PF) had the same distribution as the externally exposed rosettes in conventionally frozen, alkaline-treated membranes (Fig. 7 a and b). Because these membranes almost exclusively contained the AchR peptides, which, in addition, are transmembrane (56, 57), these inner membrane particles thus belong to the AchR protein.

Redistribution of the AchR Rosettes in the Plane of the Membrane after Phospholipid Addition

When phospholipids were mixed with the untreated membrane fragments, the receptor rosettes remained in clusters interspaced by smooth areas corresponding to lipid domains
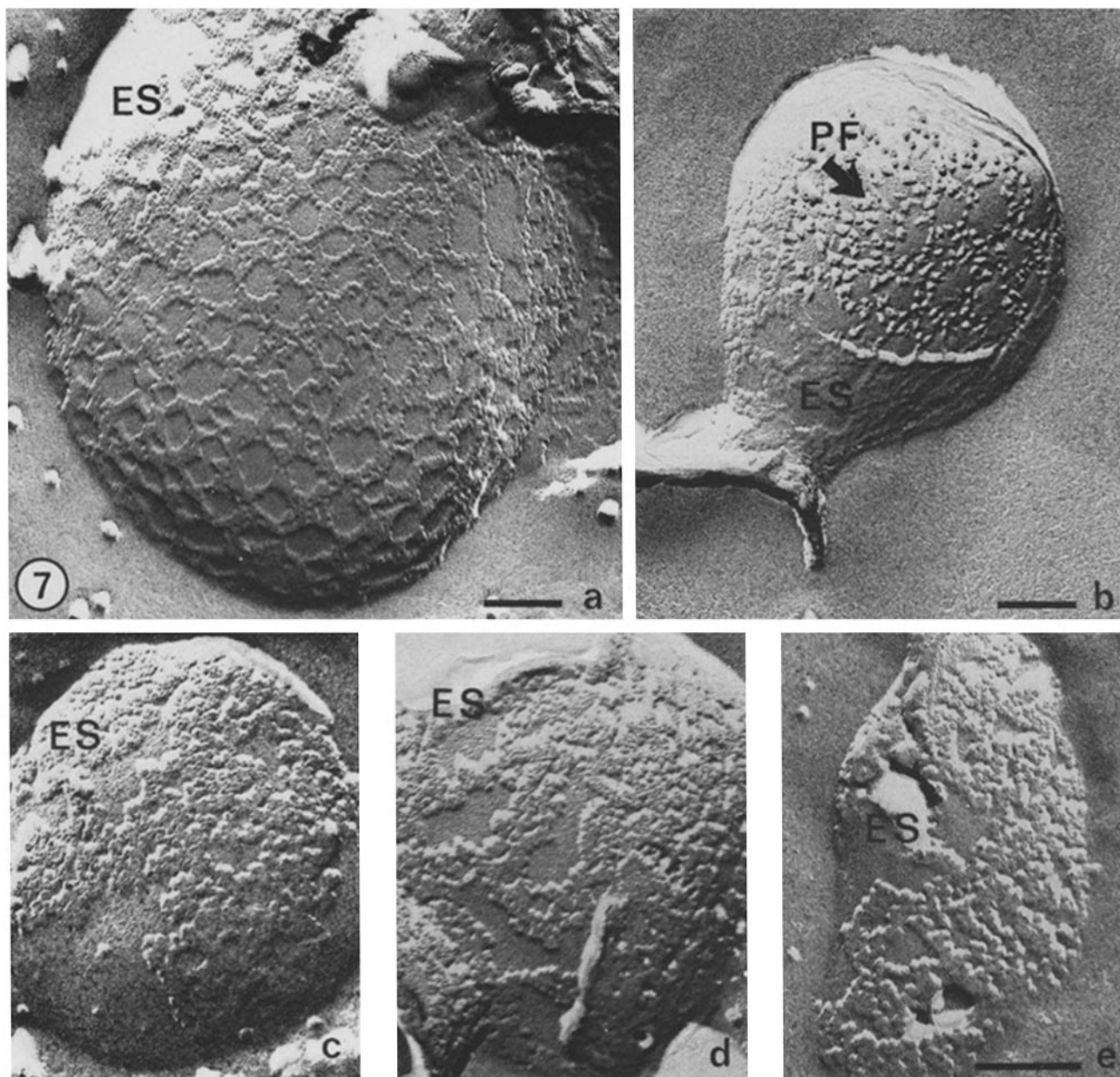

FIGURE 7   Freeze-fracture and freeze-etched aspects of alkaline-treated membrane fractions. (a) Conventionally frozen membrane fraction showing the redistribution of the rosettes on ES. (b) Occasionally, fracture plane PF was revealed, displaying a similar meshwork of inner membrane particles. (c, d, and e) Rapidly frozen samples of alkaline-treated membrane fractions. No redistribution of the rosettes is observed. Bars, 0.1 μm. a and b: × 120,000. c, d, and e: × 160,000.
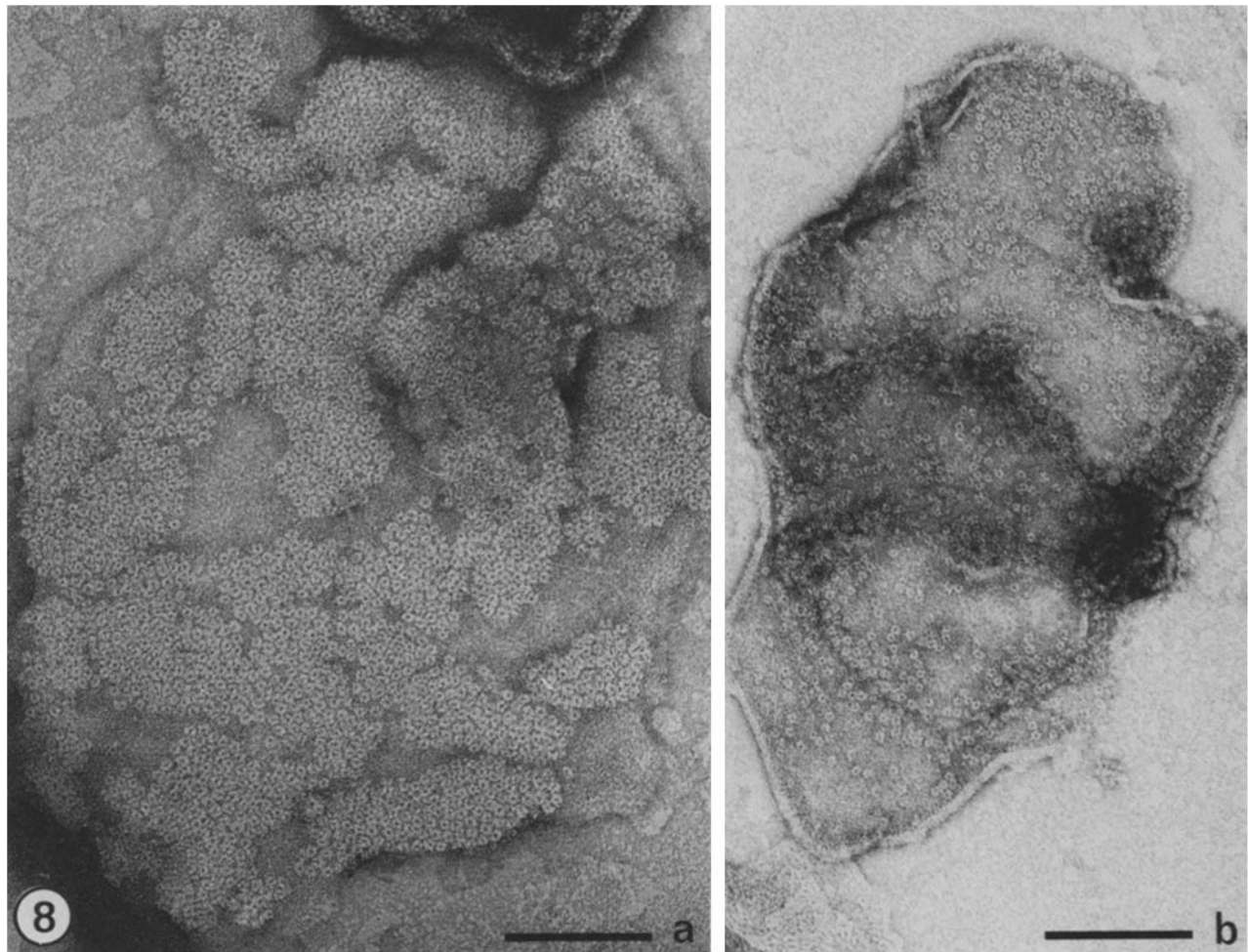
FIGURE 8 Negatively stained membranes after fusion with phospholipids. (a) Native AchR-rich membranes. After fusion with PC vesicles, large protein-free lipidic areas appear, but the receptor rosettes stay in clusters. 20–40% of the population of membrane fragments show this modified structure, indicating that fusion is not a random process. (b) Alkaline-treated membranes after fusion with PC vesicles. Note the dispersion of the acetylcholine receptor rosettes on the total surface of the membrane, indicating a lateral diffusion associated with both the extraction of the 43,000 mol wt protein and the addition of phospholipids. Bars, 0.1 μm. × 200,000.

(Fig. 8a). On the other hand, addition of the same phospholipids to the alkaline-treated membranes led to a dispersion of the rosettes within the plane of the membrane (Fig. 8b). In both cases, between 20 and 40% of the membrane vesicles (on the basis of the number of rosettes per μm² of membrane surface) appeared fused with lipids.

DISCUSSION

Comparison between the subsynaptic membrane in situ observed either by freeze-fracture (1, 11, 13, 28, 44), freeze-etching (28), or by tannic acid impregnation (51) and subfractions enriched in AchR-rich membranes (1, 11, 13, 51) revealed the same characteristic structural component: the receptor rosettes budding at the external surface and cytoplasmic condensations on the inner face of the asymmetric membrane. On the other hand, after purification, double rows of paired rosettes, consistently observed in situ, most often disappeared and the cytoplasmic condensation became unevenly distributed. The role of proteolysis in these structural alterations seemed limited because fragments prepared either following the conventional procedure (52) or in the presence of protease inhibitors disclosed similar structures.

Alkaline-extraction at 4°C removed completely the 43,000 mol wt component of the excitable membrane, but preserved the main functional properties of the membrane. After tannic acid impregnation, the cytoplasmic condensations adherent to the inner face of the membrane disappear after pH 11 treatment. Accordingly, the 43,000 mol wt protein would be a, or the, component of these "postsynaptic densities". An unambiguous demonstration of this point is, however, lacking. An immunocytochemical investigation at the electron microscopic level would be required. This interpretation is, however, consistent with the observation that the 43,000 mol wt protein is an extrinsic protein which resists proteases in intact vesicles (under conditions in which receptor peptides are digested), becomes degraded when the vesicles are opened (57), and thus is associated with the inner face of the subsynaptic membrane.

Freeze-fracture and freeze-etching experiments also revealed an important modification in the distribution of the AchR rosettes following alkaline extraction which can be interpreted as resulting from a redistribution of mobile receptor molecules when submitted to slow freezing.

Similar reorganization of membrane particles has been reported to occur in other biological or even artificial membranes as a consequence of phase separation of the lipid and protein

components (26, 32, 50). As expected, this secondary redistribution of the rosettes was no longer observed at a more rapid freezing rate.

Fusion of the AchR-rich membranes with lipid also shows a dispersion of the receptor rosettes only after pH extraction. These observations of the increase in the lateral mobility of the AchR molecules in the plane of the membrane revealed by electron microscopy are consistent with observations by electron spin resonance spectroscopy (45) or phosphorescence depolarization (36), which show an increase in rotational mobility of the AchR molecules under the same conditions. They are also consistent with the observation that, without causing any significant inactivation of the AchR molecule, alkaline extraction increases its susceptibility to heat denaturation (49).

From this ensemble of structural and dynamic data, it appears that the immobilization of the AchR protein in *Torpedo* isolated subsynaptic membrane results from the interaction of the extrinsic 43,000 mol wt protein with the cytoplasmic face of the membrane. The insensibility of the native membrane AchR to lipid phase modifications, as manifested by the absence of lateral diffusion upon fusion with phospholipids, also suggests that protein-lipid interactions do not play a critical role in the stabilization process. On the other hand, protein-protein interactions, particularly between the 43,000 mol wt protein and the transmembrane polypeptides of the AchR oligomers, appear essential to maintain the structural stability of the postsynaptic membrane domain. In this respect, the 43,000 mol wt protein would have a function similar to that of the spectrin-ankyrin system of the erythrocyte membrane (see references in reference 37).

Recent evidence showing that *in situ* the receptor protein exists mainly in its heavy form (see references in references 15 and 30) allows further speculation on the mechanism of receptor immobilization. For instance, one could hypothesize that the 43,000 mol wt protein creates a bridge between neighboring AchR doublets to form the ribbonlike assemblies of paired rosettes disclosed in freeze-fracture experiments (11, 28).

Several groups have reported the presence of filaments from the cytoskeleton in close proximity to, or even attached to, the postsynaptic densities (9, 17, 18, 20, 24). Our observations confirm a similar association of elements of the cytoskeleton with the postsynaptic domain of the *T. marmorata* electroplaque (28, 44, 51). Because these filaments disappear in purified AchR-rich membrane fractions, their attachment to the membrane should be rather loose. The chemical nature of these filaments is not known, but it has been reported that actomyosin filaments form a transcellular network within the cytoplasm (2, 33). PAGE and immunochemical data unambiguously indicate that the 43,000 mol wt protein is not actin (53). Like ankyrin (7, 21) or α-actinin (29), it may represent attachment sites between intrinsic membrane proteins and cytoskeletal components.

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