Evidence for a skeleton in acetylcholine receptor-rich membranes from *Torpedo marmorata* electric organ

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1. INTRODUCTION

In *Torpedo* electroplaque, the subsynaptic membrane of the electromotor synapse is coated on its cytoplasmic face by electron dense material (referred to as postsynaptic 'densities' or condensations) which becomes apparent in the electron microscope after tannic acid-glutaraldehyde fixation [1–5,7] but also after more conventional fixation with glutaraldehyde [6]. This structure disappears [2,7] after in vitro alkaline extraction of purified subsynaptic membranes under conditions which selectively remove nonreceptor polypeptides [8], and in particular the extrinsic and internal [9,10] 43 000 polypeptide(s) [11–13].

Two-dimensional gel analysis of the 43 000 polypeptides (named *v* [14]) reveals a dominant component which focusses at alkaline pH and two minor ones which focus at more acidic pH [15]. Of these components, named [13] respectively v_1, v_2 and v_3, only v_1 does not appear in the cell cytoplasm [13] and remains firmly bound to the membranes after repeated purification (Saitoh, unpublished). A first indication for a structural role of the 43 000 protein(s) came from the demonstration that removal of these proteins by pH 11 extraction results in an enhanced thermal susceptibility of the ACh-receptor which returns to normal after addition of the alkaline extract [16]. Morphological and spectroscopic observations further show that, upon alkaline stripping, the membrane-bound ACh-receptor experiences much faster rotational and lateral diffusion than in the native membranes [2,17–20]. Also, alkaline treatment facilitates the attack of the ACh-receptor peptides by proteases [21]. The stabilization and immobilization of the ACh-receptor in the purified membranes was thus tentatively interpreted as resulting from the interaction of the transmembrane [9,10,22–24] polypeptides of the ACh-receptor with the peripheral 43 000 protein(s) on the cytoplasmic face of the membrane [1,2,17,18,20,25–27]. This interaction could also account for one, or several, of the following events known to occur during the ontogenesis of the subsynaptic membrane: the clustering of the ACh-receptor, its *metabolic* stabilization and/or its change of channel opening time [16,18,28].

In this Letter, we report that after crosslinking of the v_1 polypeptide a filamentous network appears after Triton X-100 extraction of ACh-receptor rich membranes isolated from *Torpedo marmorata* electric organ. This structure strikingly resembles the skeleton recently described in the erythrocyte membrane [29,30].

2. MATERIALS AND METHODS

2.1. *Crosslinking of the non-receptor polypeptides and polyacrylamide gel electrophoresis*

2.1.1. Preparation of the ACh-receptor rich membranes

ACh-receptor rich membranes were purified

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from freshly dissected *Torpedo marmorata* membranes as described previously [15] using a buffer designed to inhibit endogeneous proteolytic activity (P_i buffer: 50 mM Tris–HCl, pH 7.5 (20°C), 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 5 U/ml aprotinin, and 5 μg/ml pepstatin). Membranes were stored in liquid nitrogen at 15 mg protein/ml until use.

2.1.2. Crosslinking of the 43 000 ν_1 non-receptor peptide

Membranes 30 μg/ml were incubated for 30 min, in the dark, at room temperature with 200 μM *para*-azidophenacyl bromide (PAB) from Pierce. Samples were then illuminated for 5 min at 350 nm, at room temperature and under constant agitation by magnetic stirring, using the 200 W Osram Xenon Hg lamp of a Jobin Yvon spectrofluorimeter (slit width 10 nm). Samples treated and not treated, illuminated and not illuminated were then centrifuged for 15 min in the dark in an Eppendorf minifuge.

2.1.3. One-dimensional polyacrylamide gel electrophoresis

Samples were dissolved in 25 μl of SDS sample buffer (40% glycerol, 10% SDS, 0.25 M Tris–HCl, pH 6.8, 0.01% Bromophenol blue, 10% β-mercaptoethanol).

SDS polyacrylamide gels (10% acrylamide) were prepared as described previously [11] using 1.1 mm thick slabs. Gels were stained in 0.5% Coomassie Brilliant blue R 250, destained and scanned with a Vernon gel scanner.

2.1.4. Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional gel electrophoresis was performed according to O'Farrell [31] with the following modifications. First dimension: isoelectric focussing gels were prepared using a mixture of 1/5 Ampholine 3–10 and 4/5 Ampholine 4–9. To the lower 0.01 M H_3PO_4 solution were added 2% Triton X-100 and 9 M urea. The basic ethanolamine solution was supplemented with 4.5 M urea and 2% Triton X-100 to prevent diffusion of urea and NP40 out of the gel.

2.2. *Electron microscopy*

2.2.1. Thin sectioned preparations

Membrane samples (~1 mg protein) were resuspended in 10 ml 0.2% Triton X-100, 0.2 M NaCl for 10 min at room temperature, centrifuged (Rotor 50 Ti Beckman 20 000 rev./min, 15 min) and washed two times with 0.2 M cacodylate buffer. The Triton X-100 extracted membranes were fixed with 2% glutaraldehyde (TAAB Laboratories, Reading, England), 1% tannic acid (Mallincrodt Analytical Reagent, St. Louis MO, USA) in 0.1 M cacodylate (pH 7.2) for 1 h at 4°C, post-fixed with ice-cold OsO_4 in 0.1 M cacodylate buffer (pH 7.2), and finally embedded in Vestopal W (Martin Jaeger-Vesenaz/Geneva, Switzerland). Thin (white to grey) sections were doubly stained with uranyl acetate and lead citrate [32].

2.2.2. Negative staining

Membrane fragments were attached to carbon films prepared according to Fukami and Adashi [33] before exposing them to the detergent (0.2% Triton X-100, 0.2 M NaCl) for 1–5 min. Grids were then floated onto several drops of 0.1% ammonium acetate and negatively stained with 1% uranyl acetate.

In other preparations, KI treatment of the Triton X-100 extracted membranes was sometimes performed on grids (0.6 M KI) for 5–10 min.

Observations of all specimens were made with a Philips 400 electron microscope operating at 80 kV and fitted with 50 μm objective aperture. Negatively stained pictures were taken without preexposure of the specimen to the electron beam.

3. RESULTS

3.1. *Crosslinking of ACh-receptor rich membranes*

ACh-receptor rich membranes were alkylated in the dark by the acylbromide moiety of the heterobifunctional reagent PAB and submitted to polyacrylamide gel electrophoresis in SDS. As a result, the mobility of the 43 000 band increased: it migrated close to the α chain of the ACh-receptor (fig.1Ab). On the other hand, alkylation followed by ultraviolet illumination at 350 nm caused the disappearance of most of the 43 000 band which accumulated at the top of the stacking gel. Two faint high molecular weight polypeptide bands (H.M.), which did not enter the separating gel, disappeared as well (fig. 1Ad). Gel scans (not shown)

confirmed that actin and the α, β, γ and δ chains of the receptor did not change, but that the 43 000 band decreased as a result of the crosslinking.

Two-dimensional electrophoresis revealed that the only species of the 43 000 dalton polypeptides which disappeared after crosslinking was the ν_1 form (figure 1Bb).

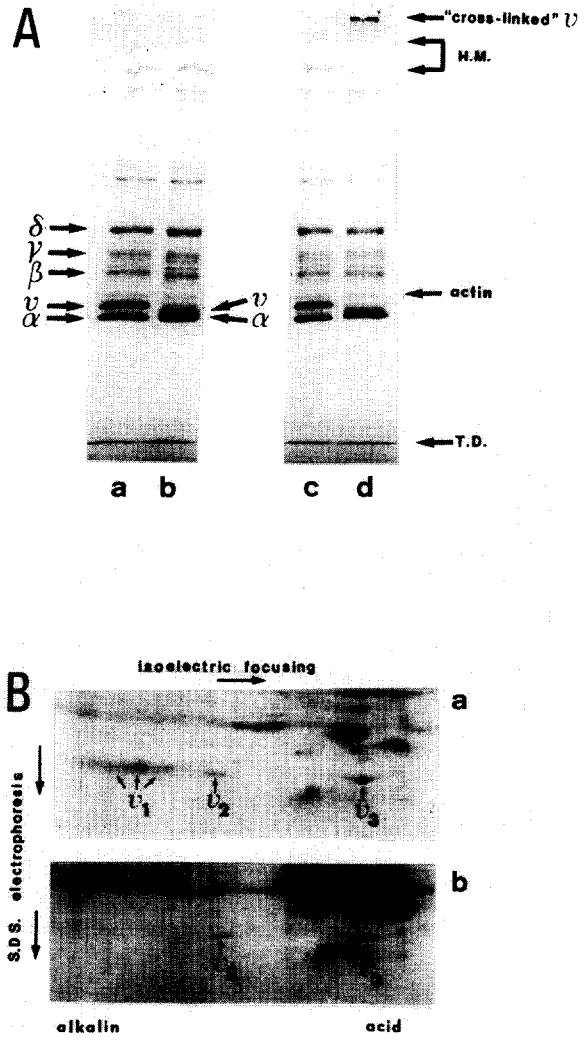


Fig.1. (A) Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of ACh-receptor rich membrane fragments from *Torpedo marmorata*. Coomassie blue staining of the 10% polyacrylamide gel. Tracks a, b (left): non-irradiated samples. Tracks c, d (right): UV irradiated samples. (a) 30 μg protein used as control. (b) 30 μg protein, PAB 200 μM. (c) 30 μg protein used as irradiated control. (d) 30 μg protein, PAB 200 μM. (B) Coomassie blue staining of two dimensional gels of ACh-receptor-rich membranes treated with PAB before (a) and after (b) UV irradiation. (a) 100 μg protein, PAB 200 μM, non-irradiated. (b) 100 μg protein, PAB 200 μM, 5 min irradiation at 350 nm.

3.2. *Ultrastructure of the crosslinked membrane fragments*

In agreement with previous work [1–4,7], after tannic-acid, glutaraldehyde fixation, resin embedding and thin sectioning, characteristic submembrane condensations were observed in preparations of isolated ACh-receptor rich membranes. After Triton X-100 extraction of either native or PAB-fixed membranes, amorphous and filamentous material persisted with remnants of membrane fragments (fig.2a and b). Anastomosed filaments sometimes comprising globular material, sometimes attached to membrane elements, were often visible. The dimensions of the network in general appeared smaller than the diameter of the native membrane fragments, possibly as a result of a collapsing of the structure during the embedding procedure.

Negatively stained preparations provide further structural details. Triton X-100 extracted native fragments yielded dispersed vesicles in which however few filamentous elements were visible (fig.2c and d). On the other hand, PAB-treated fragments frequently disclosed en-face views of the 'stabilized' meshwork (fig.3). Depending on the local conditions on the grid (this aspect was poorly controlled) the meshwork either conserved the rounded shape of the original vesicles or became dis-

Fig.2. Electron micrographs of thin-sectioned and negatively stained, Triton X-100 extracted ACh-receptor-rich membrane fragments. Triton X-100 extracted samples observed on thin sections after tannic-acid–glutaraldehyde–OsO_4 fixation. In both native (a) and PAB-treated (b) membranes, amorphous and filamentous material is observed (arrows). Remnants of membrane elements are also seen (large arrow) × 100 000. After negative staining (c and d), Triton X-100 extracted native vesicles dispersed on grid leave scattered rosettes or small membrane fragments. Filaments resembling those frequently observed after PAB-fixation (see next figure) are seldom encountered in such preparations (arrow in d) × 100 000. Bars represent 0.1 μm.

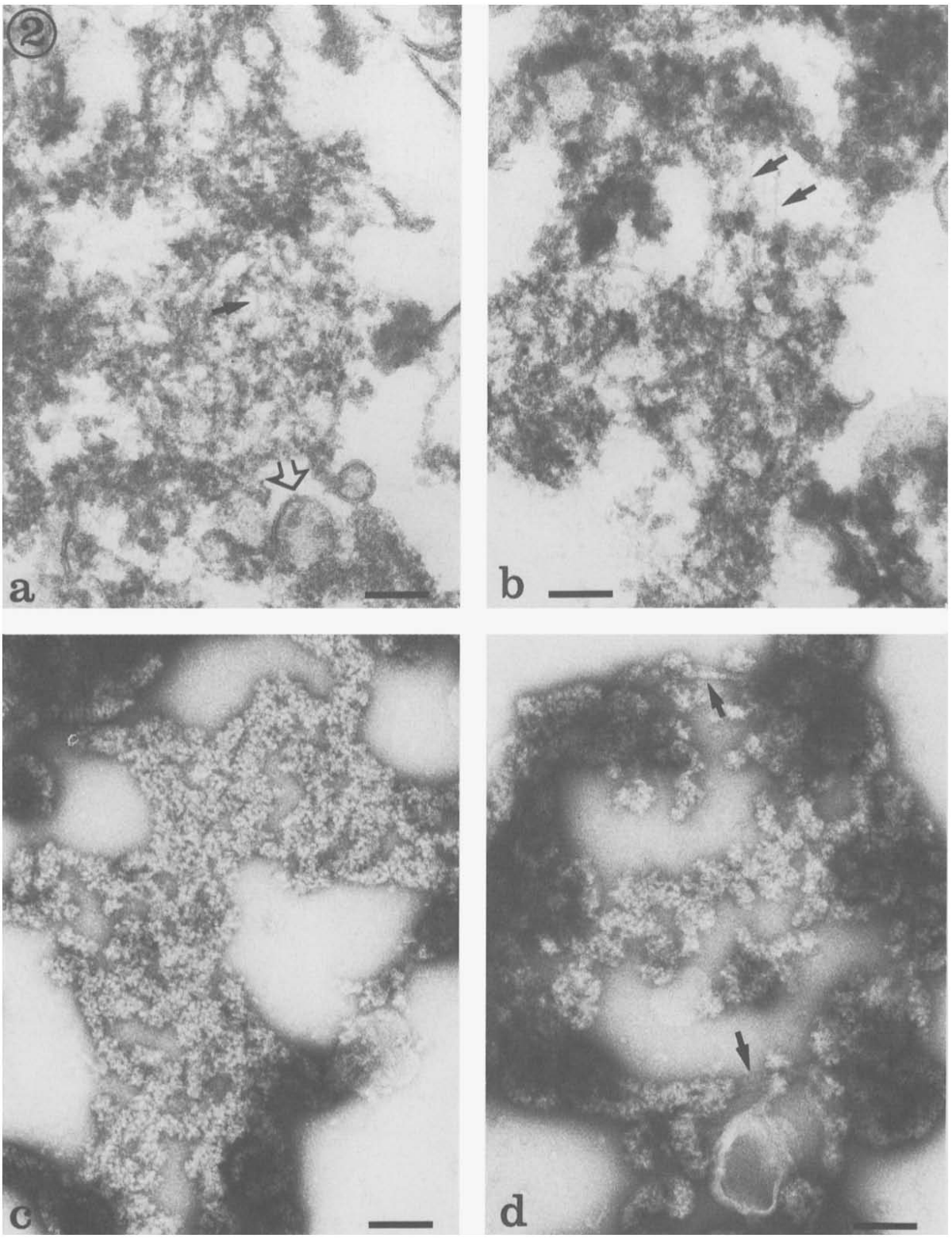
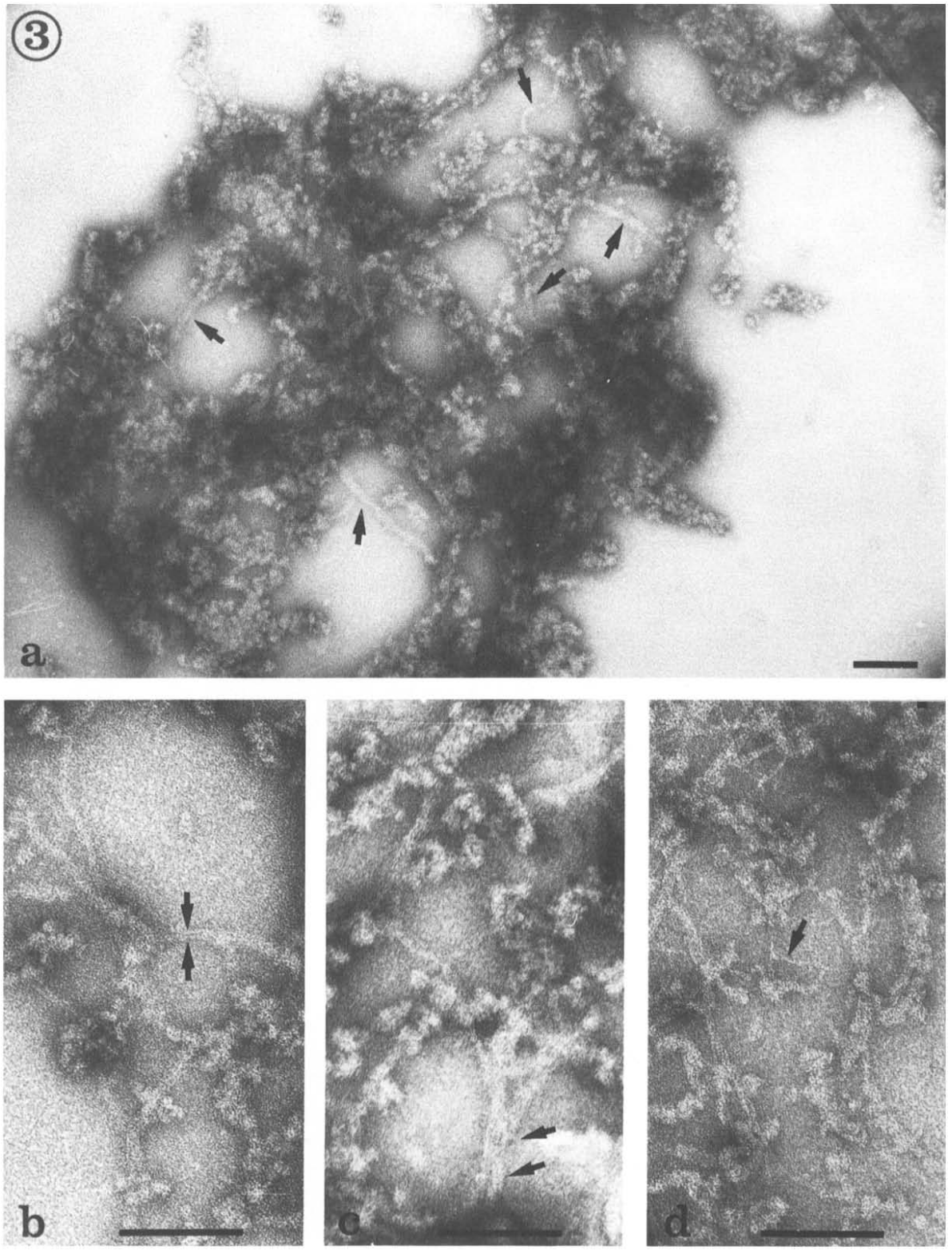


Fig.2(a–d).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a
b
c
d

persed over a large surface. In this last instance, the skeletal meshwork was the most evident and appeared as a reticulum of curved filaments of various sizes. The width of the filaments varied from 4–5 nm to an upper limit of 16–18 nm. In some areas, very thin filaments (4–6 nm) (fig.3d) were observed; in others, split or multifibrillar threads were seen (fig.3b and c). Globular entities, probably including ACh-receptor molecules, were often observed in association with the filaments. Attempts to visualize naked filaments more consistently (i.e., by a more thorough extraction of the membrane) were unsuccessful. The filaments forming the sub-membrane meshwork resisted K_i treatment (see Materials and Methods) under conditions where actin filaments would depolymerize. After alkaline extraction, under conditions known to extract nearly all the peripheral proteins, and in particular the ν proteins, we were unable to visualize even remnants of the skeleton structure after crosslinking with PAB (not shown).

4. DISCUSSION

A large body of ultrastructural and biochemical observations disclose that, in many systems [34,35], a submembrane network underlies the plasma membrane on its cytoplasmic face and characteristic components of the cytoskeleton have been identified in this detergent insoluble framework [36]. At the synapse, cytoplasmic condensations and/or filaments are found adherent to the postsynaptic membrane [37–39] and a typical cytoskeleton complex has been described at this level in guinea pig anteroventral cochlear nucleus [40].

The data presented in this Letter are consonant with these observations: we show that a filamentous network remains associated, at least partially, with fragments of the postsynaptic membrane after purification from *T. marmorata* electric organ. This skeleton appears rather labile since, on one hand, it is consistently observed only after crosslinking with the heterobifunctional reagent PAB, in particular when negative staining is used. Under these conditions of fixation, no substantial crosslinking of the ACh-receptor polypeptides (α, β, γ and δ subunits) or of actin (systematically present in purified fractions although in small quantities [41]) occurred. On the other hand, the data show that the stabilization depends on, or is related to, the crosslinking of the 43 000 dalton band. Bidimensional gel electrophoresis of the crosslinked membranes indicated that only ν_1 was crosslinked. This observation fits with the recent finding that ν_1 is associated with the postsynaptic membrane, while ν_2 and ν_3 have a more diffuse cellular distribution [13].

The biochemical identification of the structural elements of the meshwork still appears difficult: the observed filaments exhibit important polymorphism and cannot be identified strictly on morphological grounds. Most of them are still observed after treatment of the meshwork with 0.6 M K_i and thus do not represent actin microfilaments. We cannot rule out however that, although actin seems to remain unaffected by the crosslinking agent, this motility protein contributes, either in a polymerized form, or as monomers, to the structural framework associated with the ACh-receptor rich membranes. On the other hand, the fact that the structural integrity of the meshwork is influenced by the specific crosslinking of the ν_1 protein suggests that this protein is involved in the structure. The possibility that other components contribute to the network has to be considered since high molecular weight proteins (H.M. fig.1A) were also crosslinked simultaneously with the ν_1 peptide.

Globular entities are always found in contact with the meshwork, even in extensively extracted samples. Most of this material likely represents ACh-receptor molecules although their typical rosette-like appearance is not always evident. A direct demonstration of the association of the ACh-

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Fig.3. Electron micrographs of Triton X-100 extracted, PAB-treated and negatively stained membrane fragments. After PAB fixation and Triton X-100 extraction, a filamentous organization becomes visible (arrows in a) × 100 000. Detailed views in which single-layered areas displayed a reticulum of filaments (b–d). The filaments observed have a variable width (4–18 nm). Multifibrillar or split filaments are observed (arrows in b and c). A variable number of globular elements (probably ACh-receptor rosettes) are always encountered attached to the meshwork, even after extensive Triton X-100 extraction. × 200 000. Bars represent 0.1 μm.

receptor with the underlying cytoskeleton is still lacking in *Torpedo* electroplaque, in spite of the fact that the modulation of receptor mobility in the membrane clearly depends on the presence of the internal and peripheral v_1 protein [2,17–20]. In other systems (myotubes in culture), it was recently shown that upon maturation, patched receptors which present a reduced lateral mobility [42] resist Triton X-100 extraction and thus become strongly bound to the cytoskeleton while diffuse receptors do not [43].

A cytoskeleton organization of the subsynaptic domain has also been described in deep-etched rotary shadowed replicas of rapidly frozen samples [39,40]. The postsynaptic membrane appears lined by a thin network in which very thin filaments are sometimes recognized [40]. Cytoplasmic actin has also been detected in the subneural cytoplasm [44] by immunocytochemistry. Finally, it is worth mentioning that in the red blood cells a cytoskeleton membrane complex which includes a limited number of interconnected proteins, actin [45] in particular, displays structural features comparable to the network described in the present paper [29,30].

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REFERENCES

[1] Cartaud, J. (1980) in: Ontogenesis and Functional Mechanisms of Peripheral Synapses (Taxi, J. ed) pp. 199–210, Elsevier Biomedical, Amsterdam, New York.
[2] Cartaud, J., Sobel, A., Rousselet, A., Devaux, P.F. and Changeux, J.P. (1981) J. Cell Biol. 90, 418–426.
[3] Sealock, R. (1980) Brain Res. 199, 267–281.
[4] Sealock, R. (1982) J. Cell Biol. 92, 267–281.
[5] Sealock, R. and Kavookjian, A. (1980) Brain Res. 190, 81–93.
[6] Rosenbluth, J. (1975) J. Neurocytol. 4, 697–715.
[7] Sobel, A., Heidmann, T., Cartaud, J. and Changeux, J.P. (1980) Eur. J. Biochem. 110, 13–33.
[8] Neubig, R.R., Krodel, E.K., Boyd, N.D. and Cohen, J.B. (1979) Proc. Natl. Acad. Sci. USA 76, 690–694.
[9] Wennogle, L.P. and Changeux, J.P. (1980) Eur. J. Biochem. 106, 381–393.
[10] Saint-John, P.A., Froehner, S.C., Goodenough, A. and Cohen, J.B. (1982) J. Cell Biol. 92, 333–342.
[11] Sobel, A., Weber, M. and Changeux, J.P. (1977) Eur. J. Biochem. 80, 215–224.
[12] Sobel, A., Heidmann, T., Hofler, J. and Changeux, J.P. (1978) Proc. Natl. Acad. Sci. USA 75, 510–514.
[13] Gysin, R., Wirth, M. and Flanagan, S.D. (1981) J. Biol. Chem. 256, 11373–11376.
[14] Karlin, A., Damle, V., Hamilton, S., McLaughlin, M., Valderrama, R. and Wise, D. (1979) in: Advances in Cytopharmacology (Ceccarelli, B. and Clementi, F., eds), Vol. 3 pp. 183–188, Raven, New York.
[15] Saitoh, T.H., Changeux, J.P. (1980) Eur. J. Biochem. 105, 51–62.
[16] Saitoh, T.H., Wennogle, L.P. and Changeux, J.P. (1979) FEBS Lett. 108, 489–494.
[17] Rousselet, A., Cartaud, J. and Devaux, P.F. (1979) C.R. Acad. Sci Paris 289, 461–463.
[18] Barrantes, F.J., Neugebauer, W.Ch. and Zingsheim, H.P. (1980) FEBS Lett. 112, 73–78.
[19] Lo, M.M.S., Garland, P.B., Lamprecht, J. and Barnard, E. (1980) FEBS Lett. 111, 407–412.
[20] Rousselet, A., Cartaud, J., Devaux, P.F. and Changeux, J.P. (1982) EMBO J. 1, 439–445.
[21] Klymkovsky, M.W., Heuser, J.E. and Stroud, R. (1980) J. Cell Biol. 85, 823–838.
[22] Strader, C.D. and Raftery, M.A. (1980) Proc. Natl. Acad. Sci. USA 77, 5807–5811.
[23] Anderson, D.J. and Blobel, G. (1981) Proc. Natl. Acad. Sci. USA 78, 5592–5602.
[24] Froehner, S.C. (1981) Biochemistry 20, 4905–4915.
[25] Barrantes, F.J. (1982) J. Cell Biol. 92, 60–68.
[26] Froehner, S.C., Gulbrandsen, V., Hyman, C., Jeng, A., Neubig, R. and Cohen, J.B. (1981) Proc. Natl. Acad. Sci. USA 78, 5230–5234.
[27] Rousselet, A., Cartaud, J. and Devaux, J. (1981) Biochem. Biophys. Acta 648, 169–185.
[28] Changeux, J.P. (1981) The Harvey Lectures 75, 85–254.

[29] Nermut, M.V. (1981) Eur. J. Cell Biol. 25, 265–271.
[30] Timme, A. (1981) J. Ultrastr. Res. 77, 199–209.
[31] O'Farrel, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[32] Reynolds, E.S. (1963) J. Cell Biol. 17, 208–212.
[33] Fukami, A. and Adashi, K. (1965) J. Electron Microsc. 14, 112–118.
[34] Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232.
[35] Asnur, Z. and Geiger, B. (1981) J. Mol. Biol. 153, 361–380.
[36] Ben Ze'ev, A., Dueer, A., Solomon, F. and Penman, S. (1979) Cell 17, 859–865.
[37] Couteaux, R. and Pecot-Dechavassine, M. (1968) C.R. Acad. Sci. 266, 8–10.
[38] Ellisman, M.H., Rash, J.E., Staehelin, L.A. and Porter, K.R. (1976) J. Cell Biol. 68, 752–774.
[39] Heuser, J.E. and Salpeter, S.R. (1979) J. Cell Biol. 82, 150–173.
[40] Gulley, R.L. and Reese, T.S. (1981) J. Cell Biol. 91, 298–302.
[41] Strader, C.D., Lazarides, E. and Raftery, M.A. (1980) Biochem. Biophys. Res. Comm. 92, 365–373.
[42] Axelrod, D., Ravdin, P., Koppel, D.E., Schlessinger, J., Webb, W.W., Elson, E.L. and Podleski, T.R. (1976) Proc. Natl. Sci. USA 73, 4594–4598.
[43] Prives, J., Fulton, A.B., Penman, S., Daniels, M.P. and Christian, C.N. (1982) J. Cell Biol. 92, 231–236.
[44] Hall, Z.W., Lubit, B.W. and Schwartz, J.H. (1981) J. Cell Biol. 90, 789–792.
[45] Branton, D., Cohen, C.M. and Tyler, J. (1981) Cell 24, 24–32.