

Ultrastructural Localization of the M_r 43,000 Protein and the Acetylcholine Receptor in *Torpedo* Postsynaptic Membranes Using Monoclonal Antibodies

ROBERT SEALOCK, BARNABY E. WRAY, and STANLEY C. FROEHNER

Department of Physiology and The Neurobiology Program, University of North Carolina, Chapel Hill, North Carolina 27514;
Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756

ABSTRACT Four mouse monoclonal antibodies (mabs) were shown by immunoblotting procedures to recognize the major, basic, membrane-bound M_r 43,000 protein (43K protein) of acetylcholine receptor-rich postsynaptic membranes from *Torpedo nobiliana*. These mabs and a mab against an extracellular determinant on the acetylcholine receptor were used to localize the two proteins in electroplax (*Torpedo californica*) and on unsealed postsynaptic membrane fragments at the ultrastructural level. Bound mabs were revealed with a rabbit anti-mouse Ig serum and protein A-colloidal gold. The anti-43K mabs bound only to the cytoplasmic surface of the postsynaptic membrane. The distributions of the receptor and the 43K protein along the membrane were found to be coextensive. Distances between the membrane center and gold particles were very similar for anti-receptor and anti-43K mabs (29 ± 7 nm and 26 to 29 ± 7 to 10 nm, respectively). These results show that the 43K protein is a receptor-specific protein having a restricted spatial relationship to the membrane. They thus support models in which the 43K protein is associated with the cytoplasmic domains of the receptor molecule.

The major proteins of isolated postsynaptic membranes from electric tissue include the subunits of the acetylcholine receptor and a nonreceptor protein having an apparent molecular weight of approximately 43,000 (43K protein)¹ (6, 9, 19, 27). The 43K protein has stimulated considerable interest because it is a cytoplasmic, peripheral membrane protein (17, 22, 30) that has been localized to postsynaptic regions in electric tissue (6, 9, 18, 27) and the neuromuscular junction (7).² Its functions may include anchoring of the receptor against lateral diffusion (1, 5, 15, 21). However, unlike the several cytoskeletal proteins that accumulate at the junction and may also participate in receptor anchoring (3, 10), the 43K protein seems to have no known counterpart in other systems, and very little is known concerning the mechanisms by which it functions.

The postsynaptic location of the 43K protein has prompted wide speculation that it has receptor-specific functions mediated by its association with the receptor. This hypothesis predicts that the 43K protein, like the receptor (26), should

occur on the juxtaneural postsynaptic membrane of the electroplax, but should be essentially absent from the membrane in the deep portions of the postsynaptic invaginations. Hence, a careful comparison of the postsynaptic distributions of the two proteins constitutes an essential test of the hypothesis. The available immunofluorescence measurements (7)² have not had the resolution necessary for such a test. The ultrastructural morphology of the membrane suggests that the two distributions are coextensive (5, 23–25), but thus far, putative morphological correlates of the 43K protein have been identified only by indirect means. And while a recent immunoelectron microscopic study (18) confirmed that the 43K protein occurs on the juxtaneural membrane, it did not contain data on unambiguously identified invaginations or show the receptor distribution directly. In this study, we used monoclonal antibodies to localize the 43K protein relative to the receptor and to the membrane bilayer in fixed electroplax (*Torpedo californica*) and in unsealed membrane fragments at the ultrastructural level. The results support a model in which the 43K protein is receptor-associated.

MATERIALS AND METHODS

Production of Monoclonal Antibodies: The preparation of mouse monoclonal antibody (mab) 139A has been described previously (8).

¹ Abbreviations used in this paper: 43K protein, the major membrane-bound M_r 43,000 protein of isolated postsynaptic membranes; mab, monoclonal antibody; 2-D, 2-dimensional.

² S. C. Froehner, manuscript submitted for publication.

Mabs of the IgG class that recognize the 43K protein were prepared in the Dartmouth laboratory by similar methods applied to a lithium diiodosalicylate extract of acetylcholine receptor-rich postsynaptic membranes isolated from electric tissue of *Torpedo nobiliana* (19). The preparation of these antibodies will be described in detail elsewhere.²

Immunoblots: Nitrocellulose replicas of 2-dimensional (SDS and isoelectric focusing) polyacrylamide gels (2-D gels) were reacted with antibodies and analyzed by autoradiography as described (8, 19).

Immunocytochemistry: Small pieces of electric tissue, taken from three specimens of *T. californica*, fixed by perfusion with Ringer solution supplemented with a paraformaldehyde-based fixative (16), were subjected to immunochemical labeling in the Chapel Hill laboratory according to a general protocol that will be described in detail elsewhere.³ Briefly, the pieces were mounted on nylon sticks, immersed for 90 s in 95% ethanol at -20°C to render cell membranes permeable to immunochemicals (11), equilibrated with buffer (a modified *Torpedo* Ringer containing 4% [wt/vol] radioimmunoassay grade bovine serum albumin [Sigma Chemical Co., St. Louis, MO]), then treated successively at room temperature with mabs or mouse IgG at various concentrations, rabbit anti-mouse IgG, and protein A adsorbed to 3-9 nm colloidal gold (20), and prepared for electron microscopy. Two tissue pieces in each sample were sectioned (silver-gold to gold sections) at multiple sites on their surfaces, and regions of cells having various intensities of specific labeling and of background were photographed for comparison to each other and to cells reacted with nonspecific mouse IgG in place of the mabs.

Crude, predominantly unsealed postsynaptic membrane fragments were prepared from electric tissue (*T. californica*) by two manual (Teflon-in-glass) homogenizations in 25% sucrose-1 mM ZnCl₂ and a high speed (Virtis 45) homogenization in 20 mM Tris(hydroxymethyl) aminomethane, pH 7.4, followed by fractionation on 25-50% sucrose gradients containing 50 μM ZnCl₂.³ Samples of the crude membranes were centrifuged onto the bottoms of polyvinylchloride microculture wells, fixed in 0.2% glutaraldehyde, cleared of aldehydes with sodium borohydride (29), and immunochemically labeled in essentially the same way as electric tissue.

For quantitative analysis of the distances between gold particles and the membrane, micrographs were made of all stretches of transversely sectioned bilayer in a given field on poststained ultrathin (gray) sections. The coordinates of membranes and gold particles were entered via a graphics data tablet into a computer that divided the membrane lengths into 5-nm bins and calculated the distance from each particle to the nearest bin.

RESULTS

Characterization of Mabs

At least three major proteins having molecular weights near 43,000 have been identified in preparations of receptor-rich membranes. This requires that the specificity of mabs be identified by the use of 2-D gels. Fig. 1A shows a Coomassie Blue-stained 2-D gel of a preparation of receptor-rich membranes similar to those from which the 43K protein was extracted for immunization. An immunoblot prepared from a companion gel using mab 1098C (Fig. 1B) shows that the antibody recognizes only the three most basic proteins at M_r 43,000. These proteins have been shown previously to be isoelectric variants of a single protein (9, 19). Their basic isoelectric point range and their near total absence (>90%) from the cytosol (unpublished data) identify these proteins as the major basic, membrane-bound 43K protein described in detail by Porter and Froehner (19). This protein is probably identical to the protein designated ν_1 by Gysin et al. (9). Mab 1098C is unreactive with all other proteins in the membrane preparation, including the less basic protein of slightly lower molecular weight (arrow in Fig. 1A), which is probably the predominately soluble protein called ν_2 by Gysin et al. (9) (and which may be creatine kinase [2]), and the acidic M_r 43,000 protein, which is probably actin (19).

Three additional anti-43K mabs (1132E, 1114A, and 1097B) having the same specificity were also characterized in this way.

³ B. E. Wray and R. Sealock, manuscripts in preparation.

Immunocytochemistry

Each of the four anti-43K mabs was used to localize the 43K protein in small pieces of electroplax that had been fixed in situ.

Fig. 2 shows a portion of the innervated face of an electroplax labeled with mab 1098C. The juxtaneural postsynaptic membrane is heavily and nearly homogeneously labeled on its cytoplasmic surface, but the membrane in the deep portions of the postsynaptic invaginations is unlabeled, or is labeled in small patches that are difficult to distinguish from background labeling. Labeling of the extracellular surface of the postsynaptic membrane and of all nonpostsynaptic structures including the noninnervated face (not shown) varied from light to undetectable and was qualitatively indistinguishable from that obtained with equivalent concentrations of nonspecific mouse IgG (not shown). Nonspecific IgG also did not label the cytoplasmic surface of the juxtaneural membrane.

This pattern of labeling was seen with all four anti-43K mabs over a 10-fold range of mab concentration, and in regions that had been more extensively damaged by the permeabilization procedure than in Fig. 2. Hence, high densities of specific binding sites for these anti-43K mabs are, like the receptor (26), confined to the juxtaneural postsynaptic membrane in *Torpedo* electric tissue. We have directly compared the distributions of the two proteins by labeling tissue pieces with anti-43K mabs supplemented with mab 139A, which recognizes an extracellular determinant on the receptor (8). The distributions were found to be very similar (Fig. 3A), although most frequently either colloidal gold field could extend significantly further than the other (Fig. 3, B and C).

The variability shown in Fig. 3 could easily arise if one or more of the immunochemical reactions could not go to completion under the conditions of these experiments. This is likely because of impediments to diffusion such as the cytoplasm, the basal lamina, and the overlying nerve terminals, and most samples did, in fact, show some evidence of incomplete reaction. To increase the degree of reaction, we took advantage of the fact that postsynaptic membranes from electric tissue, like plasma membranes from other cells (4, 28), form sheets, unsealed tubes, and some sealed vesicles when the tissue is homogenized in the presence of ZnCl₂.³ Since most membrane fragments prepared in this way are separated from the basal lamina, full access of immunochemicals to both sides of the membrane is assured. Small samples of the fragments were centrifuged onto the bottoms of polyvinylchloride microculture wells and fixed lightly to prevent vesiculation and sealing of the fragments before labeling. Extensive examination of samples labeled on either the receptor or the 43K protein established that mab 139A and anti-43K mabs bind almost exclusively to the convex and concave surfaces, respectively (Fig. 4, A and B). Nonspecific mouse IgG labels neither side (not shown). In the majority of sections through postsynaptic fragments labeled simultaneously with mab 139A and anti-43K mabs, both sides are fully labeled (Fig. 4B). A minority of the postsynaptic fragments shows significant interruptions in the receptor field that may arise from the in situ boundaries between receptor-rich and receptor-poor membrane on the sides of the invaginations. At such interruptions, the variability in the relative extents of the two gold particle fields is much less than that in tissue pieces. In general, the two fields stop together (Fig. 4, D-G) or the

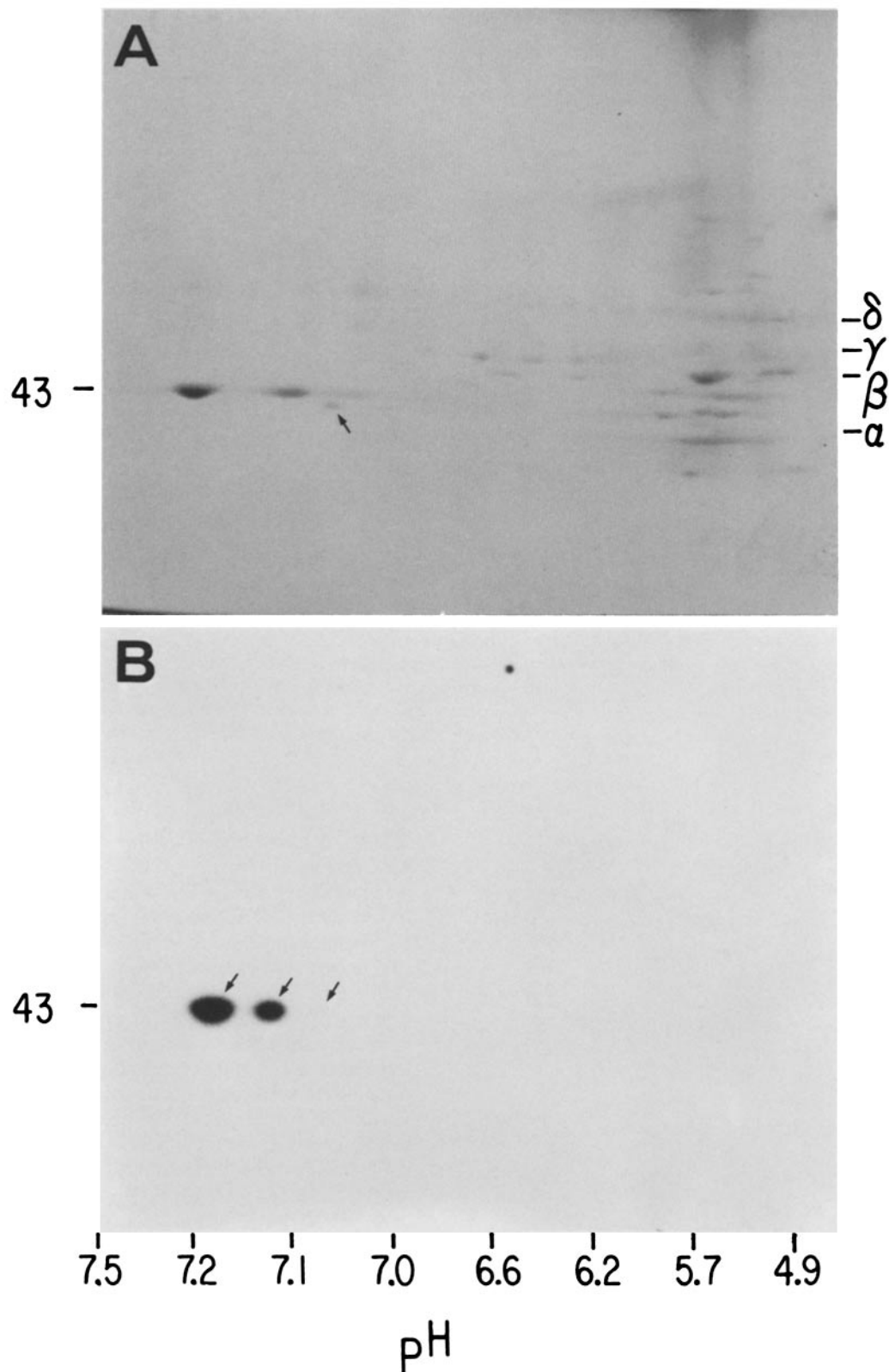


FIGURE 1 2-D gels of purified receptor-rich membranes. (A) Stained with Coomassie Blue. Two major and one minor spots at M_r 43,000 (43) on the basic (*left*) side of the gel are isoelectric variants of a single protein (19), probably corresponding to ν_1 of Gysin et al. (9). A minor spot lying just below the three variants (arrow) may correspond to ν_2 , although we have no other evidence for such an identification. (B) Autoradiograph of a mab 1098C-labeled immunoblot of a companion gel. There are three reactive spots (arrows), corresponding to the three Coomassie blue-stained variants in A. The molecular weight scale was calibrated on similar gels using the subunits of the isolated receptor (Greek letters on the right ordinate) (see reference 19). The pH gradient was measured on similar gels.

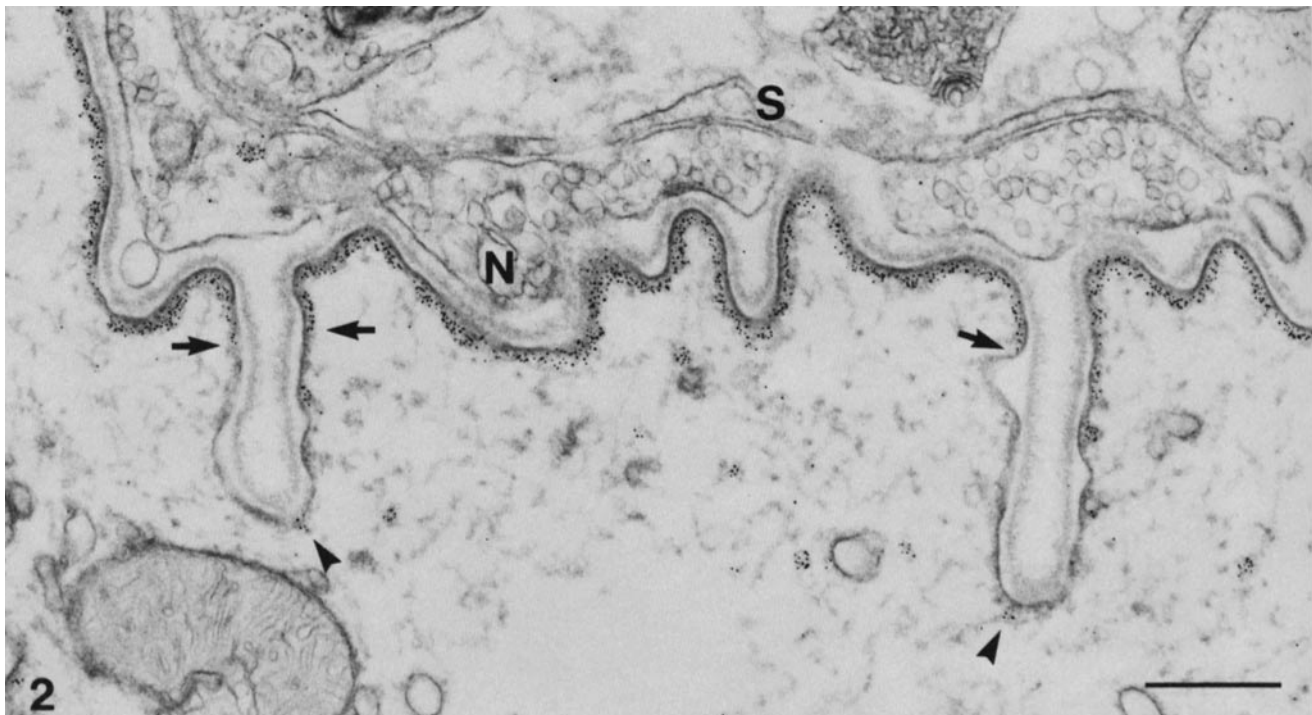


FIGURE 2 A portion of the innervated face of an electroplax labeled with anti-43,000 mab 1098C. The colloidal gold field on the cytoplasmic surface of the postsynaptic membrane descends into the invaginations, then stops (arrows). Occasional patches can be seen on the deeper portions of the invaginations (arrowheads). Labeling in all other regions is at background levels. The clumps of colloidal gold in this micrograph appear to be due to variably occurring, nonspecific clumps of first antibody; we observed them with nonspecific mouse IgG, and they were often absent from well labeled regions. *N*, nerve terminals; *S*, Schwann cells. Bar, 500 nm. $\times 38,000$.

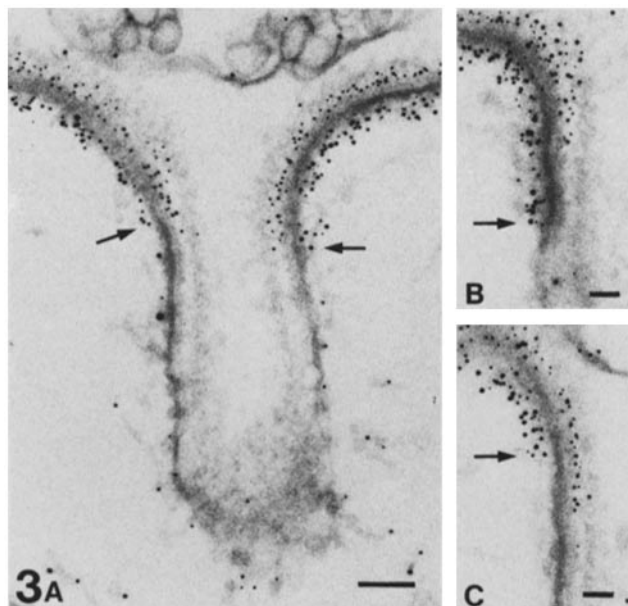


FIGURE 3 Postsynaptic invaginations in cells labeled simultaneously with anti-receptor mab 139A and anti-43,000 mab 1132E. Arrows indicate the points at which the *intracellular* field of label terminates. (A) The two colloidal gold fields stop together. Bar, 100 nm. $\times 77,000$. (B) The intracellular field extends 125 nm further into the invagination than the extracellular field. Bar, 50 nm. $\times 86,000$. (C) The extracellular field extends 100 nm beyond the intracellular field. Bar, 50 nm. $\times 86,000$.

receptor field may extend slightly beyond the 43K field (Fig. 4, C, E, and F).

It can also be seen in Fig. 4 that the colloidal gold particles linked to anti-43K and anti-receptor mabs tend to lie in narrow bands at about the same distance from the membrane. Exceptions, such as the labeling of what appears to be a short filament in Fig. 4E, do occur, but whether they reflect 43K protein dissociating from the membrane, antibody aggregates, or specifically labeled filaments is not clear. They are minor features in any event; the overall visual impression was confirmed by a preliminary quantitative analysis of the distances separating particles from the bilayer center. The distance-frequency histograms for mabs 139A and 1097B on membrane fragments are similar to each other (Fig. 5 and Table I) and to those for mab 1098C on membrane fragments and mabs 1097B and 139A on tissue pieces (Table I). (The presence of small antibody aggregates prevented a meaningful analysis with mabs 1132E and 1114A.) These results suggest that the binding sites for anti-43K and anti-receptor mabs have similar relationships to the membrane.

DISCUSSION

In this study, we have used four anti-43K mabs and one mab against the receptor to localize the two proteins relative to each other in *Torpedo* postsynaptic membranes. The anti-43K mabs recognize at least three distinct sites on the 43K molecule,² so that it is unlikely that the 43K protein occurs in unlabeled regions of electric tissue but in forms that are

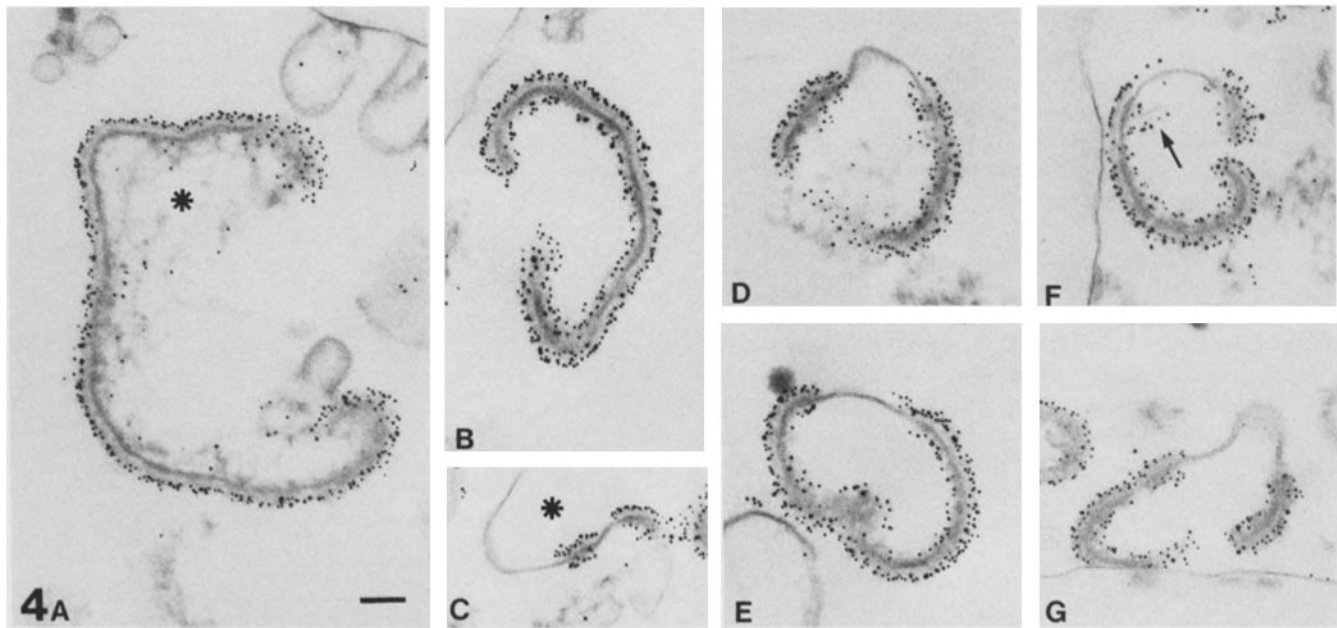


FIGURE 4 Postsynaptic membrane fragments. (A) A large fragment with retained cytoplasmic filaments (*), labeled with mab 139A. The inner surface and several nonpostsynaptic membranes (top of the micrograph) are labeled at background levels. (B) A fragment labeled simultaneously with mabs 139A and 1097B. Both sides are nearly fully labeled. (C–F) Fragments which contain significant interruptions in the receptor field. (C–F) Fragments labeled with mabs 139A and 1097B. In C, the asterisk indicates the extracellular surface, identified from information outside the area shown. In F, the arrow indicates an apparent labeled filament of unknown origin. (G) A portion of a fragment labeled with mabs 139A and 1098C. Bars: (A, B, and D–F) 100 nm; (C and G) 120 nm. (A, B, and D–F) $\times 59,000$; (C and G) $\times 49,000$.

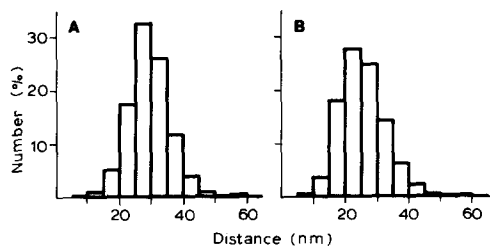


FIGURE 5 Distance-frequency histograms for membrane fragments labeled simultaneously with mabs 139A (shown in A) and 1097B (in B). These data are also shown in Table I.

not reactive with any of the four mabs. Our results, therefore, confirm previous findings that the 43K protein is confined to the postsynaptic region in electric tissue (7, 18) and the neuromuscular junction (7). More importantly, by focusing on boundaries between receptor-rich and receptor-poor regions at the ultrastructural level, we have demonstrated directly that the postsynaptic receptor is very closely accompanied by the 43K protein. We were unable to determine whether the distributions of the two proteins are precisely coextensive in situ, but this can probably be explained by the difficulty of achieving full immunochemical reaction in tissue pieces. The results obtained with postsynaptic membrane fragments, where physical impediments to diffusion have been eliminated, suggest that the two distributions are coextensive to within the resolution of the immunochemical procedure (perhaps 25 nm, or three times the diameter of a single receptor monomer). Although interruptions in the receptor fields of isolated fragments can not be identified with in situ boundaries with certainty, these results agree with the tentative conclusion established from ultrastructural studies of

TABLE I
Membrane—Gold Distances

	mabs*		
	Membrane fragments		Tissue
	139A + 1097B	139A + 1098C	139A or 1097B
	nm		
d_o	29 ± 6.7 (1471)	29 ± 6.4 (472)	27 ± 7.6 (612)
d_i	26 ± 7.4 (713)	29 ± 9.9 (283)	22 ± 9.2 (635)

Distances were measured from the apparent bilayer center to gold particles on the extracellular (d_o) and intracellular (d_i) surfaces. The parentheses enclose the number of particles measured.

* Membrane fragments or tissue pieces were labeled with the mabs listed.

isolated postsynaptic membranes (5, 23, 25) and the neuromuscular junction (24), in which putative morphological correlates of the 43K protein were found to be coextensive with the morphologically identified receptor. It appears, then, that the acetylcholine receptor and the 43K protein are coextensively distributed at a molecular level of resolution over most, and probably all, of the juxtaneural postsynaptic membrane.

Binding sites for mab 139A must occur in a narrow band lying somewhere between 2 and 8 nm from the bilayer center, since the extracellular portion of the receptor extends 5–6 nm beyond the membrane surface (13). The similar widths and means of the distance-frequency histograms obtained with anti-43K and anti-receptor mabs, therefore, clearly suggest that the 43K protein must lie close to, and in a restricted spatial relationship to, the membrane. These immunocytochemical results support models in which the 43K protein is associated with the receptor. In particular, they appear to confirm the model developed from tannic acid-mediated negative contrasting of postsynaptic membranes (24, 25), in

which the 43K protein lies among or just subjacent to, the cytoplasmic ends of the receptor.

The precise mechanism by which the 43K protein may contribute to the stabilization and organization of receptor accumulations remains unclear. Its removal from conventionally isolated membranes that contain few proteins other than the receptor and the 43K protein leads to increased mobility of the receptor (1, 15, 21). The obvious conclusion from such experiments is weakened, however, by the use of harsh conditions (pH 11) to effect the removal. In addition, receptors on isolated fragments from *T. californica*, before removal of the 43K protein, appear to be substantially less organized than on innervated face sheets or in whole electroplax (12, 14). Full receptor organization may, therefore, require the additional submembrane materials that are seen in situ (5, 23), but which are lost in conventional preparations from *T. californica* (25). In this case, the role, or one of the roles, of the 43K protein may be to serve as a link between the receptor and the submembrane material. In addition, some features of receptor organization may be imposed by mechanical aspects of the association between the basal lamina and the postsynaptic membrane. Answers to these questions must await determination of the number of receptor molecules contacted by each 43K molecule, and of the relationship between the 43K protein and other submembrane components.

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