

Cytoplasmic Surface Structure in Postsynaptic Membranes from Electric Tissue Visualized by Tannic-acid-mediated Negative Contrasting

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ABSTRACT In this study, acetylcholine receptor-rich postsynaptic membranes from electric tissues of the electric rays *Narcine brasiliensis* and *Torpedo californica* are negatively contrasted for thin-section electron microscopy through the use of tannic acid. Both outer (extracellular) and inner (cytoplasmic) membrane surfaces are negatively contrasted, and can be studied together in transverse sections. The hydrophobic portion of the membrane appears as a thin (~2 nm), strongly contrasted band. This band is the only image given by membrane regions which are devoid of acetylcholine receptor. In regions of high receptor density, however, both surfaces of the membrane are seen to bear or be associated with material which extends ~6.5 nm beyond the center of the bilayer. The material on the outer surface can be identified with the well-known extracellular portion of the receptor molecule. A major portion of the inner surface image is eliminated by extraction of the membranes at pH 11 to remove peripheral membrane proteins, principally the 43,000 M_r (43K) protein. The images thus suggest a cytoplasmic localization of the 43K protein, with its distribution being coextensive with that of the receptor. They also suggest that the 43K protein extends farther from the cytoplasmic surface than does the receptor.

The nicotinic acetylcholine receptor in electric tissue of electric rays is a transmembrane protein (40, 41, 44) which is localized to limited, juxtaneural regions of the postsynaptic membrane (33). Biochemical preparations of the postsynaptic membrane contain the subunits of the receptor protein, one major peripheral membrane protein which is designated 43K¹ after its apparent molecular weight on denaturing gels (22, 35, 37), and a few minor proteins. Whether the latter are components of postsynaptic or contaminating membranes is not known. The 43K protein, however, has been localized to innervated regions of electric tissue (10), and it remains with receptor-containing membranes through several steps of purification (8, 37), constituting 30% or more of the total protein in the final preparations. Biochemical evidence suggests that the 43K protein,

which can be distinguished from actin by several criteria (36, 39), is associated with the cytoplasmic surface of the membrane (29, 30, 44).

Ultrastructural studies of receptor-rich membranes have provided little information concerning the location of the 43K protein. X-ray diffraction studies of membranes isolated from *Torpedo californica* have yielded markedly asymmetric electron-density profiles in which the outer (extracellular) half of the membrane is thick (6.5–8 nm), but the inner (cytoplasmic) half is thin (3–4 nm) (16, 27). The thick outer half could be ascribed to portions of the receptor molecule which are well-known to project above the surface of the bilayer (4, 7, 13, 16). The remainder of the profile was also ascribed to the receptor, although the preparations used in the study almost certainly contained the 43K protein (cf. 8, 37). In thin-section electron microscopy, the postsynaptic membrane from *T. marmorata* has also been reported to be markedly asymmetric after fixation in the presence of tannic acid (3–5, 35). On the other hand, I have shown using tannic acid that in membranes isolated from a close relative of *Torpedo* (*Narcine brasiliensis*), regions which contain the receptor at high density appear as a symmetric unit

¹ The term "43K protein" in this paper refers to membrane-bound protein material which migrates as a single band on one-dimensional polyacrylamide-SDS gels with $M_r \approx 43,000$ and is quantitatively extracted at pH 11. Although this material has behaved as a single protein species in the present experiments, evidence that it may consist of multiple species has been presented (1, 11).

membrane in which both the outer and inner laminae are unusually thick (~8 nm) (32). These images suggested that the inner surface, like the outer, may bear substantial amounts of material, although the possibility that the inner surface image arose from only small amounts of material which poststained very intensely could not be fully eliminated.

The symmetric model of the membrane can be tested in transverse views of negatively contrasted membranes, since negative contrasting reports more on the concentration of materials in a structure than on their affinity for heavy metals. In this study, I have used tannic acid (21) to prepare negatively contrasted postsynaptic membranes for thin-section electron microscopy. The impetus for this approach came from the well-known property of tannic acid to cause heavy metal buildup around microtubules, revealing them in negative contrast (42), and from observations that the receptor is sometimes negatively contrasted in electric tissue fixed in the presence of tannic acid (24; R. Sealock, submitted for publication). The method provides large numbers of transverse views which clearly show the presence of material associated with the cytoplasmic surface in membranes from both *Narcine* and *T. californica*. The material is confined to regions of high receptor density. Differences in the images obtained with native membranes and with membranes from which peripheral proteins have been removed by extraction at pH 11 suggest that the 43K protein may be a major component of this material.

MATERIALS AND METHODS

Special Materials

Live *Narcine brasiliensis* and *Torpedo californica* were obtained from Island Laboratories, Port Aransas, Tex., and Pacific Bio-Marine, Venice, Calif., respectively. Tannic acid refers to the low molecular weight tannic acid (34) from Mallinckrodt, Inc., St. Louis, Mo. (catalogue no. AR1764). Polymer-free glutaraldehyde (Polysciences, Inc., Warrington, Pa.) was used in all fixations. Millipore filters were obtained from Millipore Corp., Bedford, Mass.

Membrane Isolation and Extraction

Postsynaptic membranes were prepared from fresh electric tissue by the method of Sobel et al. (37). For alkaline extractions, the desired fractions from the last sucrose gradients were pooled, diluted with water, and centrifuged. The pellet was resuspended in water at 0°C to give a final protein concentration of 1 mg/ml. The membrane suspension was removed from ice only for rapid adjustment to pH 11 with 0.1 N sodium hydroxide, then immediately returned to 0°C, and centrifuged 10 min at 25,000 rpm in a Beckman type 65 rotor (Beckman

Instruments Inc., Spinco Div., Palo Alto, Calif.). The supernatant was combined with 1/9 vol of 0.5 M Tris, pH 7.4. The membranes were resuspended in cold 30% sucrose to their original volume.

Gel Electrophoresis

PAGE in the presence of SDS was carried out according to Laemmli (17), using gels with 8.75% acrylamide and 0.215% bis-acrylamide. Gels were stained with Coomassie Blue.

Preparation for Electron Microscopy

Membranes in sucrose (40 µg/sample) were supplemented with CaCl₂ to a final concentration of 2 mM, then fixed at 0°C by addition of cold 4% glutaraldehyde/50 mM Na phosphate/0.8% tannic acid, pH 7.3. After 1 h, the samples were diluted with 1.5% glutaraldehyde/20 mM Na phosphate/0.3% tannic acid, pH 7.3, and placed in Beckman microfuge tubes containing Epon stubs (1.8 cm long) on which were resting discs (3 mm in diameter) cut from Millipore VSWP filters. The discs were shiny side up. The stubs were prepared by polymerizing Epon in microfuge tubes, and then flattening the tops of the Epon pieces with a file. After an additional 20 min at 0°C, the tubes were centrifuged 30 min at 10,000 rpm in a Beckman SW27 rotor at 0°C. The discs, bearing a barely visible sample layer, were transferred with forceps to vials containing 100 mM Na phosphate/0.5 mM CaCl₂, pH 7.0. After washing and postfixation for 1 h in 0.8% osmium tetroxide in the same buffer, the samples were washed in water, dehydrated with graded alcohols, and infiltrated beginning with Epon:ethanol 1:1 (vol:vol). Infiltration was continued with Epon:propylene oxide 3:1 (vol:vol) and 100% Epon. Propylene oxide is avoided in the first infiltration step to prevent extensive breaking up of the sample layer due to swelling of the filter.

Blocks were sectioned until a region containing suitable amounts of negative contrasting material was found by light microscopy (see Fig. 1). Silver sections were then cut, poststained with 2% uranyl acetate in water (10 min), followed by Reynold's lead citrate (25) (10 min).

Measurements

Membranes were photographed in a Zeiss EM 10A at magnifications of 31,600 or 50,800 at 60 kV with a 60-mm objective aperture. Particularly clear, well-contrasted regions which appeared to have been transversely sectioned, as judged by a thin middle lamina, were printed at × 300,000 or × 700,000. Lines were drawn parallel to the middle lamina, along the outer and inner edges of the images. The perpendicular distances from the center of the middle lamina to the outer line (l_o) and to the inner line (l_i), and the overall width ($l_o + l_i$) were measured. Generally, only one suitable region could be found per vesicle. For extracted membranes, the inner half was measured only where single outer surface projections and detail in the inner surface image were apposed. 50–70 points were measured for native and extracted membranes.

RESULTS

Negative contrasting of postsynaptic membranes with the aid of tannic acid has been induced in three ways in this study. In

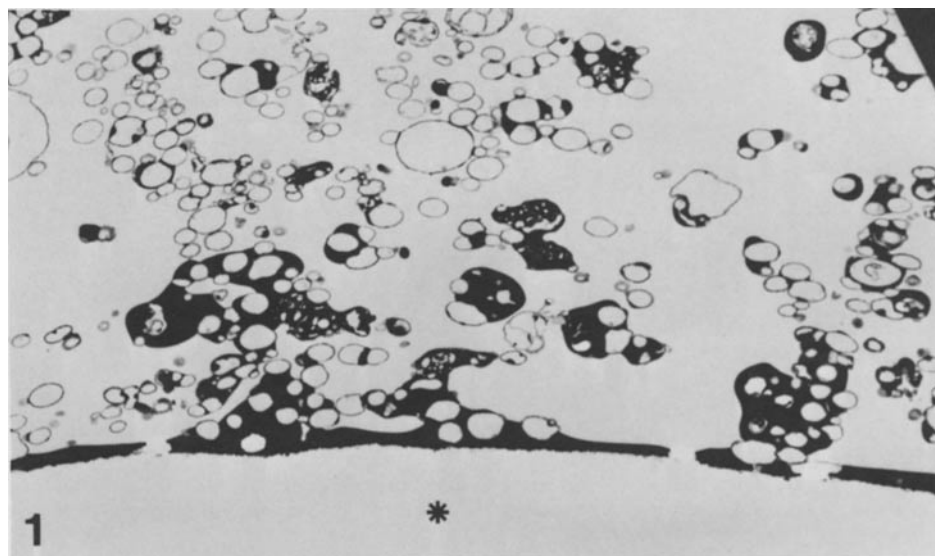


FIGURE 1 Negative contrasting material at and above the interface of a membrane sample and a Millipore VSWP filter (*). Many membrane vesicles are wholly or partially embedded in the material. Scale, 1 cm \cong 1.6 µm. × 6,100.

the first, the membranes were fixed in suspension with glutaraldehyde in buffer containing 0.3–1.0% tannic acid, pelleted, and then prepared for microscopy by conventional methods. The overwhelming majority of the membranes in such samples is positively contrasted (32). However, extensive sectioning of the sample will usually reveal one or more regions in which the positive contrasting passes subtly into negative contrasting, albeit of rather weak intensity. A stronger negative contrasting can be induced throughout such a pellet by soaking it for 1 h or more in 6–8% tannic acid in buffer before postfixation with osmium tetroxide. Even with overnight incubations, however, this method failed to give the very strong contrast seen when cellular structures such as flagella, the mitotic apparatus, and microtubules are similarly treated (42). The strongest contrasting by far appeared when we began pelleting fixed membranes onto a supporting disc cut from Millipore VSWP filters. The bulk of the sample is again positively contrasted, but at the interface of the sample and the filter are found numerous membranes which are wholly or partially surrounded by an unusual electron-opaque material which reveals them in strong negative contrast (Fig. 1).

The precise nature of this contrasting material is not known. It clearly involves an interaction between tannic acid and the filter, however. Its occurrence requires the presence of tannic acid during the centrifugation step. It does not occur in the absence of a filter, or in the second of two samples centrifuged sequentially onto a single filter and processed together. VSWP filters cause more of it to appear than do other Millipore filters such as HAWP filters, and the shiny side of the VSWP filter favors it more than the dull side. Submersion of a VSWP, but not a HAWP, filter in solutions of tannic acid, with or without the other components of the fixative, causes a prompt release

of small, filamentous particles. This chemical reaction seems to involve the surface of the filter, since it slows after several minutes and the rest of the filter remains intact even after several days. In a possible explanation for the contrasting material, membranes may bind nonspecifically to the dissolving surface, the particles of which could react with or serve as nucleation sites for large quantities of tannic acid. Subsequent reaction with osmium tetroxide would give a material which is electron dense. Its electron density is further increased by conventional poststaining.

The three methods of inducing negative contrasting lead to entirely equivalent conclusions concerning postsynaptic membrane structure. Images shown here are of samples prepared on VSWP filters as described in Materials and Methods.

Membranes derived from each of the several membrane species in electric tissue can be found associated with the contrasting material. In preparations from both *Narcine* and *Torpedo*, the majority of transversely sectioned membranes contains varying lengths of a membrane species which gives a broad image (Figs. 2 and 3). The thickness of the membrane in these regions is 13–14 nm (Table I). This image is roughly bisected by a strongly contrasted middle lamina ~2 nm in width. These membranes may also contain regions which give only thin images, similar to and in continuity with the middle lamina (Figs. 2 and 3). The remaining membranes give only thin, sharply contrasted lines. The line, the common element in all the images, is presumed to arise from the hydrophobic portion of the bilayer, while the inner and outer surface images reflect material on or associated with specialized regions of the membrane.

The two halves of the broad image are distinctly different. The outer half contains varying degrees of substructure which,

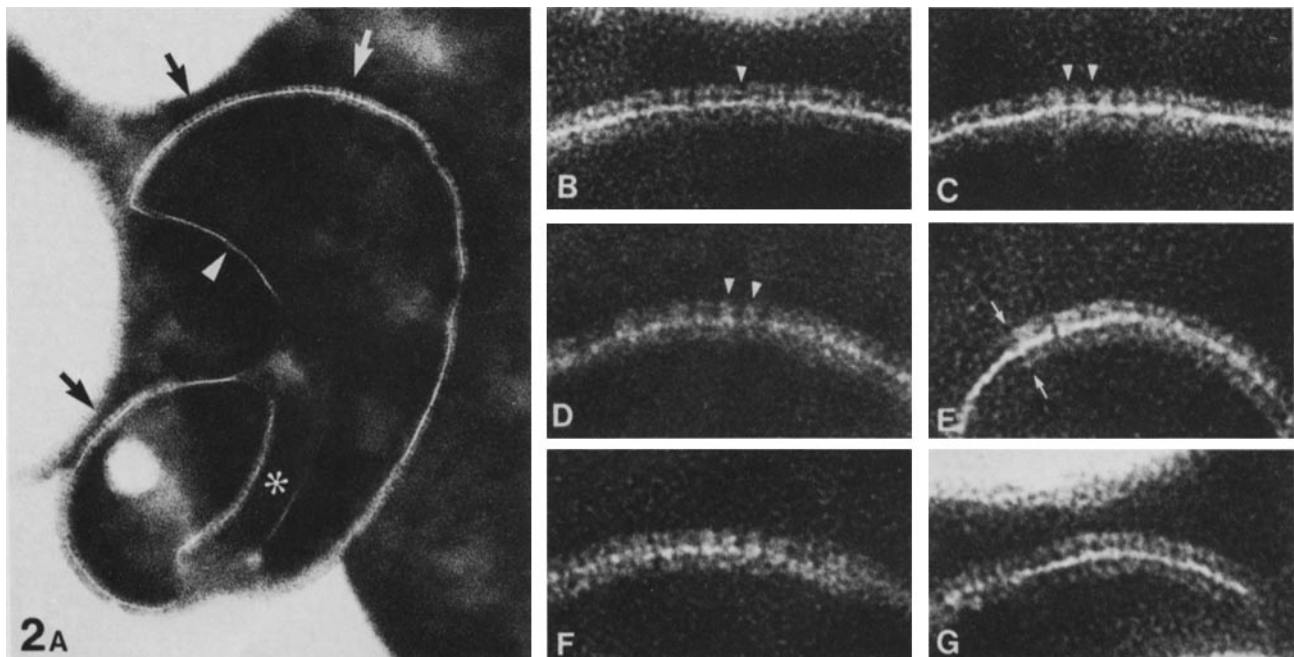


FIGURE 2 Negatively contrasted postsynaptic membranes from *Narcine*. In micrographs B–G of this figure, and in Figs. 3 B–G, 5 A, and 7 A–F, the outer membrane surface lies above the middle lamina. (A) A single vesicle in which outer surface projections can be seen clearly in three areas (arrows). It also contains thin membrane (arrowhead), and is deformed in one area (*), presumably due to interaction with the contrasting material. (B and C) Two of the areas marked by arrows in A at higher magnification. What appear to be thin bridges between adjacent outer surface projections can sometimes be seen (arrowheads). (D–G) Typical examples of the broad image in other vesicles. In D, well-defined projections in the outer surface (arrowheads) contrast with the featureless appearance of the inner surface. In E, the inner and outer surface specializations stop together (arrows). (A) 2 μm \cong 0.15 μm . \times 131,000. (B–G) 1 μm \cong 2.4 nm. \times 420,000.

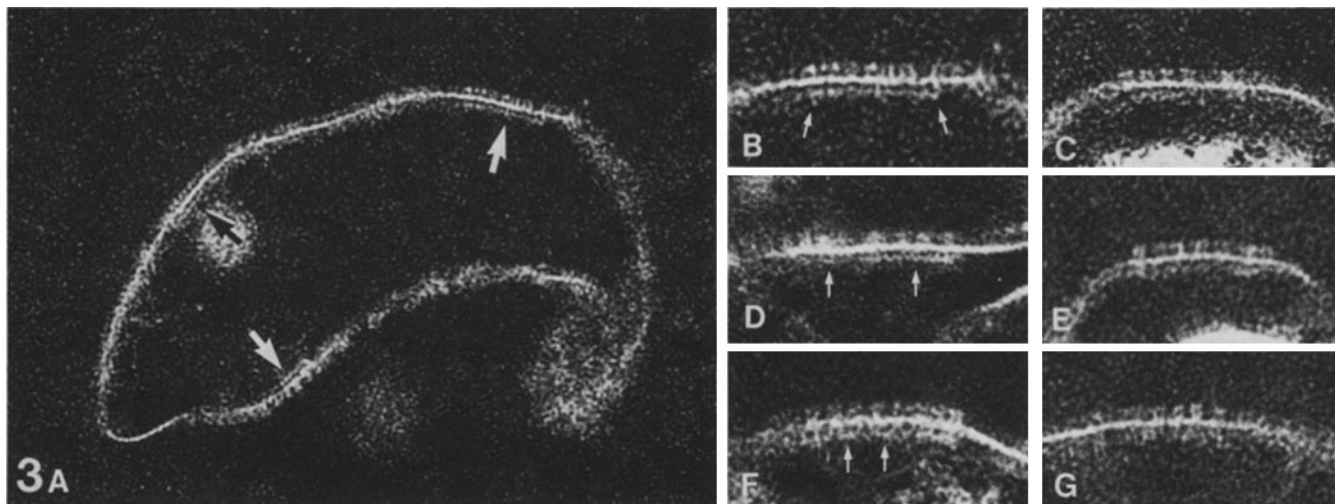


FIGURE 3 Negatively contrasted postsynaptic membranes from *Torpedo*. (A) A vesicle containing three regions in which the inner surface image appears as a distinct bar (arrows). (B) The uppermost region in A at higher magnification, with the bar indicated by arrows. (C–G) Typical views of the membrane in other vesicles. The inner surface has a wider range of appearance in membranes from *Torpedo* than from *Narcine*, from the distinct bar (D, arrows) to images with essentially no indication of the bar (G). Intermediate forms are the most common (C, E, F). Elements of the bar in F are indicated by arrows. (A) $2\text{ cm} \cong 0.09\ \mu\text{m}$. $\times 225,000$. (B–G) $1\text{ mm} \cong 3\text{ nm}$. $\times 350,000$.

TABLE I
Dimensions of Postsynaptic Membranes Obtained by
Negative Contrasting

	Control membranes		Extracted membranes (<i>Torpedo</i>)
	<i>Narcine</i>	<i>Torpedo</i>	
	nm		nm
l_o^*	6.8 ± 0.6	6.3 ± 0.6	6.5 ± 0.4
l_i^\ddagger	6.9 ± 0.9	6.4 ± 0.7	5.6 ± 0.8
$l_o + l_i$	13.7 ± 1.2	12.8 ± 1.1	12.2 ± 1.2
l_o/l_i	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.1

* The distance from the center of the middle lamina to the outermost edge of the image.

‡ The distance from the center of the middle lamina to the innermost edge of the image.

in the clearest views, consists of distinct, roughly rectangular projections sitting on the middle lamina (Figs. 2B, C, and D, and 3G). The center-to-center separation of the projections is $\sim 9\text{ nm}$. In some views, particularly of the membranes from *Narcine*, a thin bridge of material appears to connect the projections (Fig. 2C). In other views, the projections are less distinct, presumably because the structures which give rise to them are not directly superimposed through the thickness of the section. The inner half of the broad image can also have two extreme forms. In one, it appears as a rather homogeneous cloud attached to the middle lamina (Fig. 2D and F). In the second, it appears as distinct bar of material separated from the middle lamina by a thin zone which is infiltrated by contrasting material (Fig. 3B and D). This zone remains throughout a through-focus series of micrographs (not shown), and so does not arise by inappropriate use of the microscope. In addition, both forms of the image can be found in the same membrane vesicle. The first predominates in membranes from *Narcine* (Fig. 2), and the second is more common in membranes from *Torpedo* (Fig. 3). Most of the images in either case lie between these two extremes (Figs. 2E and G, and 3C and F).

Substructure within the inner surface image sometimes seems to suggest that structural elements in the inner surface may be

aligned with the structures which give rise to the projections in the outer surface image (Fig. 2C). This is clearest at the edges of the broad image, where the two halves stop together abruptly (Figs. 2E and 3F). Occasionally, significant lengths ($>50\text{ nm}$) of strongly contrasted outer surface image seem to be unaccompanied by a broad inner surface image, or are accompanied by only a weakly contrasted image. For example, this was found in 4 out of 98 negatively contrasted fragments surveyed on one grid. Broad inner surface images which are well contrasted but not accompanied by a broad outer surface image are extremely rare.

The broad image is approximately symmetrical with respect to thickness of its two halves. The perpendicular distances from the center of the middle lamina to the maximum external extent of the image (l_o) and to the maximum internal extent (l_i) are approximately the same (Table I).

The molecular origins of the outer surface image can be identified from several lines of evidence: (a) Membranes which are partially surrounded by contrasting material can be unambiguously identified as postsynaptic or nonpostsynaptic by the morphology of their positively contrasted portions (32). Only those which are recognizable as postsynaptic membranes present the broad image in their negatively contrasted portions (Fig. 4). (b) The average extension of the projections beyond the center of the middle lamina ($\sim 6.4\text{ nm}$, Table I) and their center-to-center separation ($\sim 9\text{ nm}$) are similar to values obtained by others for the membrane-bound acetylcholine receptor from *Torpedo* (1, 4, 13, 15). (c) The structure which gives rise to the projections has a central canal. This can be seen in side views of apparently single structures (Fig. 5A) and in *en face* views obtained when membranes are tangentially sectioned (Fig. 5B). The latter show distinct, roughly round units, of which the center contains contrast material and the annulus contains hints of substructure. Similar images are routinely obtained by conventional negative staining of the acetylcholine receptor protein (4, 7, 16, 23, 47). (d) Projections like those on the isolated membranes are also seen in sections of electric tissue and mouse neuromuscular junctions which have been subjected to a similar negative contrasting procedure (R. Sea-

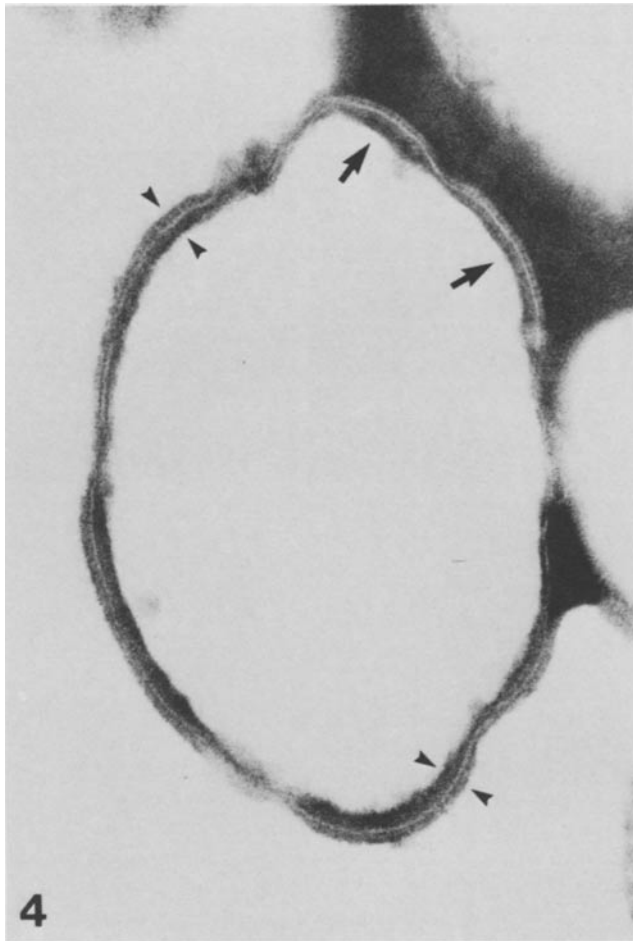


FIGURE 4 A membrane vesicle from *Narcine* which can be identified as a postsynaptic membrane by the thick, roughly symmetric image in its positively contrasted portions (32) (arrowheads). A portion of the vesicle is negatively contrasted, and shows the broad image (arrows). 1 mm \cong 3 nm. \times 154,000.

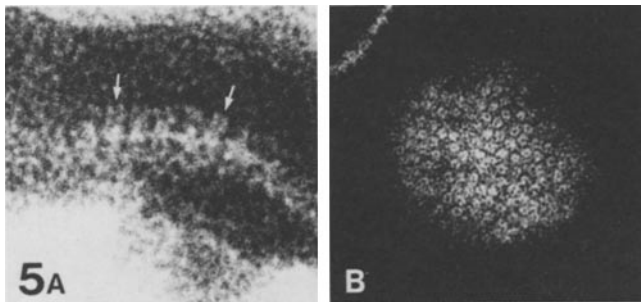


FIGURE 5 Negatively contrasted postsynaptic membranes from *Narcine* (A) and *Torpedo* (B). (A) A transverse section which appears to show single receptor molecules (arrows). (B) An *en face* view showing many rosettelike structures which have a central depression or hole. In both A and B, the indicated structures are identified as correlates of the receptor molecule by comparison to similar images obtained by negative staining (4, 7, 16, 23, 47). (A) 1 mm \cong 2 nm. \times 490,000. (B) 2 mm \cong 9 nm. \times 226,000.

lock, submitted for publication). In both tissues, they occur throughout regions known to contain the receptor at high density (9, 33), but they are absent elsewhere. (e) The receptor in these membranes and at the neuromuscular junction occurs in more or less ordered arrays at high density ($>6,000$ mole-

cules/ μm^2) separated by regions of receptor-free membrane (4, 13, 15, 32). The projections, therefore, are morphological correlates of the extracellular portions of the receptor protein with highest contrast and greatest clarity coming from receptor molecules which are superimposed in columns extending through the sections parallel to the optical axis of the microscope.

Postsynaptic membrane vesicles from electric tissue are generally found to be >90 – 95% right side out in biochemical experiments (12, 30, 44). Identification of the projections with the receptor protein provides another criterion, in addition to those established previously (32), by which this point can be checked morphologically. In this and the previous study, I have not seen a single vesicle which appeared to be inside out.

In spite of uncertainties concerning the mechanism of tannic acid-mediated negative contrasting, the contrast material seems to reveal structure in the receptor molecule in considerable detail, and to thoroughly infiltrate both membrane surfaces. One can conclude that the cytoplasmic surface of postsynaptic membranes from both *Narcine* and *T. californica* must contain or be associated with a distinct specialization which contains substantial amounts of material and is specific to regions of high receptor density.

Alkaline-extracted Membranes

Nonreceptor peptides can be removed from postsynaptic membranes by extraction at pH 11 (22), a procedure developed by Steck and Yu for removal of peripheral membrane proteins from the erythrocyte ghost (38). The images given by the membranes before and after extraction provide a route to correlation of morphology with the extracted components. Accordingly, membranes from *T. californica* were adjusted to pH 11 at 0°C in water, immediately centrifuged, and resuspended in 35% sucrose (total time at pH 11, ~ 15 min). The ^{125}I - α -bungarotoxin binding activity of the receptor was quantitatively recovered in the membrane pellet, as expected (22) (data not shown). The extract contained the 43K protein and several minor proteins (Fig. 6). As is usual in our preparations from *Torpedo*, the 43K protein was quantitatively extracted. The origins of the minor components are unknown. None has been identified as a specific component of the postsynaptic membrane, and they could also originate from several types of contaminating membranes in the preparations (32).

Alkaline-extracted membranes are strikingly different from control membranes when visualized by negative contrasting (Fig. 7). Strongly contrasted projections are still seen on the convex surface of the middle lamina. These can be identified with the receptor as before, since its extracellular portion is morphologically unaltered after alkaline extraction (15, 35). The projections are, however, somewhat less distinct than in control membranes, possibly due to a lower degree of order in the arrangement of receptor molecules in the plane of the membrane (1). The width of the outer half of the membrane is essentially unchanged (Table I). In contrast, the broad inner surface image is greatly reduced in its strength of contrasting, its clarity, and the definition of its innermost (cytoplasmic) edge, sometimes almost to the point of being absent altogether. Even when the inner surface is strongly contrasted, its innermost edge appears diffuse and indistinct (Fig. 7B). Frequently, the points in the inner surface image having the strongest contrast occur opposite outer surface projections which are distinct and well-contrasted (see Fig. 7A and F for examples). The total transmembrane width at these points is ~ 12 nm

(Table I). This is slightly less than the value for the control membrane, with the difference resulting from the thinner inner surface image, but slightly more than the value usually found for the length of the detergent-solubilized receptor molecule (11 nm) (3, 4, 35). It seems doubtful that the difference with the latter value has any significance.

The absence of a strong band for the γ subunit of the receptor protein on SDS gels (Fig. 6) suggests that the membrane-bound receptor may have undergone proteolysis by endogenous proteases during preparation of the control membranes. Such proteolysis of the receptor typically gives degradation products which are not visible on gels stained with Coomassie Blue (22, 37). These products normally do not dissociate from the molecule unless it is denatured (18), but they might dissociate under the harsh conditions of the alkaline extraction. This point was tested by centrifugation of ^{125}I - α -bungarotoxin-labeled receptor from control and extracted membranes on 5–20% sucrose gradients in the presence of Triton X-100. The sedimentation velocities of the monomer and dimer forms of

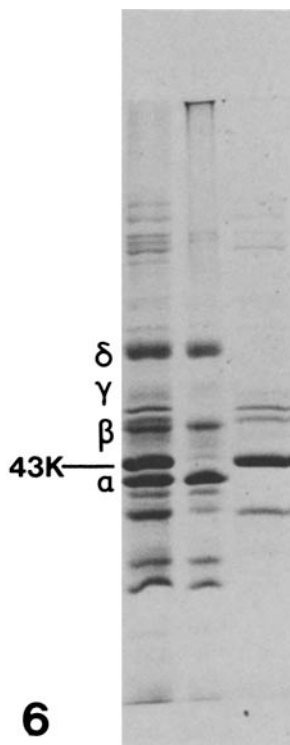


FIGURE 6 SDS gels of control membranes (1st lane), extracted membranes (2nd lane), and the supernatant from the extraction (3rd lane). The four subunits of the receptor were identified by their presence in the extracted membranes and their apparent molecular weights of 39,000 (α), 48,000 (β), 57,000 (γ), and 66,000 (δ), which are similar to published values (8, 18, 35). Some of the bands below α may be due to partial proteolysis of receptor subunits during membrane preparation (18, 44).

the receptor (6) and their relative amounts in the two samples were indistinguishable by this method, suggesting that a large loss of receptor peptides could not have contributed significantly to the change in the image after alkaline extraction.

Similar alterations in morphology were obtained with membranes from *Narcine* after alkaline extraction and with membranes from *Torpedo* after extraction with 10 mM lithium diiodosalicylate at neutral pH, a procedure which removes the same protein species as alkaline extraction (8) (data not shown).

Positively Contrasted Membranes

An early expectation in this study was that alkaline extraction of postsynaptic membranes would lead to structural alterations which could easily be detected in positively contrasted membranes. However, in contrast to the clear difference between control and alkaline-extracted membranes seen in negative contrasting, rather little difference is seen with positive contrasting. This point is illustrated here with membranes from *Torpedo* (Fig. 8), although the same result is obtained with membranes from *Narcine*. The image of the control membrane after fixation in the presence of tannic acid is similar to that previously described for *Narcine* (32): it shows distinctly thickened inner and outer laminae; the outer lamina tends to contain substructure, the inner lamina tends to stain more intensely than the outer, and the overall width of the images is similar (~18 nm) (Fig. 8A). A survey of the membrane species in the *Torpedo* electroplaque (R. Sealock, submitted for publication) shows that, as for *Narcine* (32), this morphology uniquely identifies acetylcholine receptor-rich regions. The image is rather little changed after alkaline extraction (Fig. 8B). It remains nearly symmetric with respect to thickness, although extracted membranes in which the inner lamina appears to be slightly less thick than the outer are readily found (Fig. 8B). The inner lamina continues to stain intensely and to contain very little substructure. Hence, positive contrasting fails to provide convincing evidence for the alterations in structure seen so readily in negative contrasting, at least in the absence of extensive data analysis. This result is presumably explained by the similar widths of native and extracted membranes (Table I), and by the tendency of positive contrasting to make structures appear somewhat thicker than they are in fact.

DISCUSSION

In this paper, I have made use of tannic acid to cause extensive buildup of heavy metal stains around structures, thus revealing

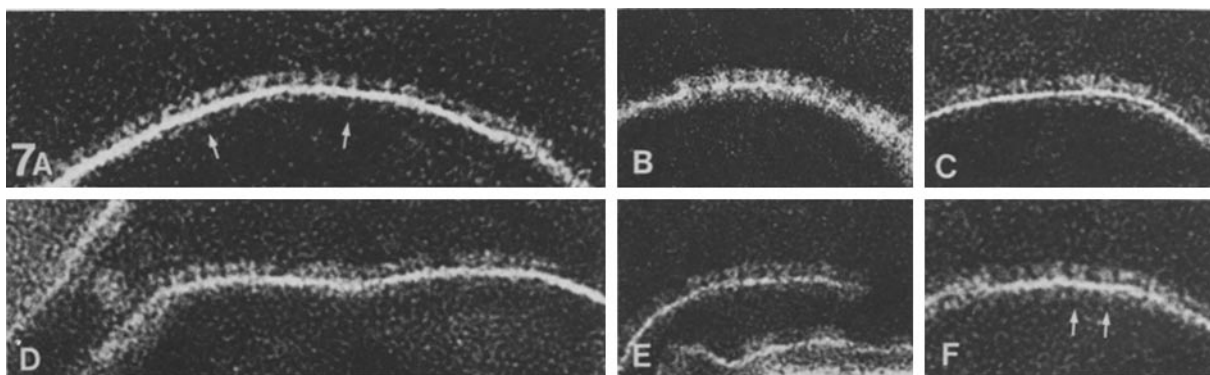


FIGURE 7 Typical views of alkaline-extracted postsynaptic membranes from *Torpedo*. The outer membrane surface can be identified by its projections, but the amount of contrasted material is greatly reduced on the inner surface. Frequently, points of strongest contrasting in the inner surface image are directly apposed to individual projections (arrows in A and F). 1 mm \cong 3 nm. \times 350,000.

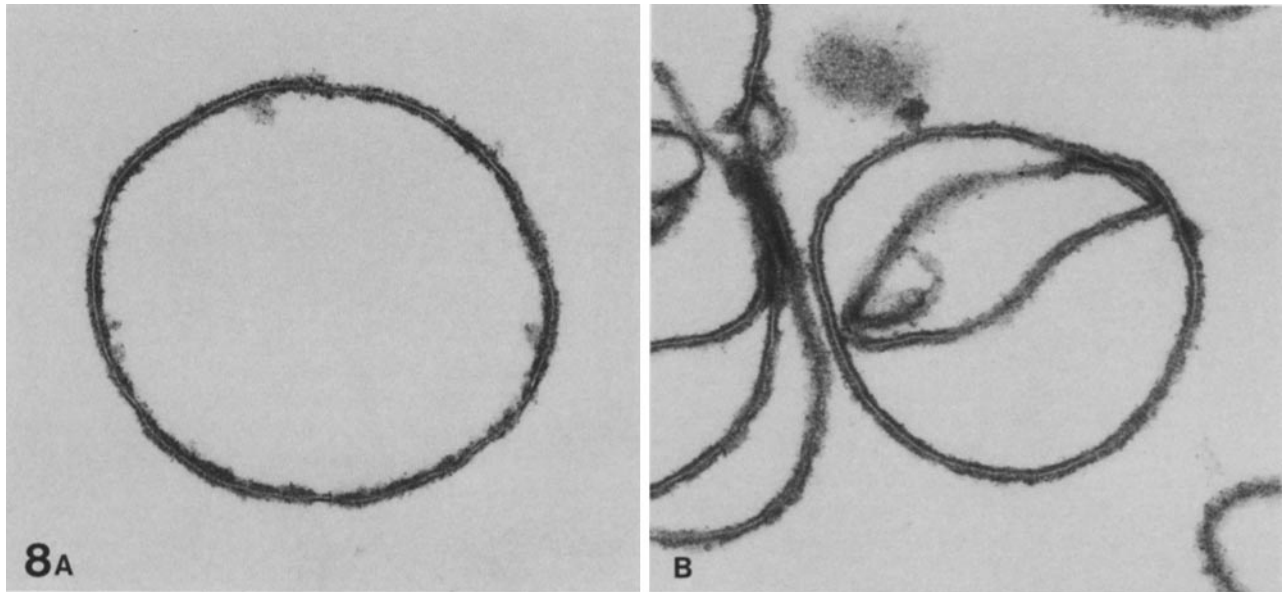


FIGURE 8 Control (A) and alkaline-extracted (B) postsynaptic membranes from *Torpedo*. These particular images were selected to indicate roughly the maximum difference between control and extracted membranes which is encountered. In spite of the short time at pH 11 and the low temperature, postsynaptic membranes are frequently opened by alkaline extraction, shown by the presence of membranes inside other membranes (B). This is never seen with control membranes. The inner lamina of the unit membrane seems to be somewhat thinner than the outer lamina in B, but not in A. 1 cm \cong 0.09 μ m. \times 110,000.

them in negative contrast. This action of tannic acid was also enhanced through a poorly understood interaction with the material of Millipore VSWP filters. In spite of the mechanistic uncertainties of this method, the images of single acetylcholine receptor molecules obtained with it suggest considerable resemblance to conventional methods of negative staining. The advantage of negative contrasting for thin-section electron microscopy over conventional negative staining was that large numbers of transverse views, in which both sides of the membrane are seen together, could be obtained readily. The disadvantage was that much of the resolution which is apparently inherent in the method was sacrificed due to superposition of contrasted structures through the thickness of the sections (\sim 60 nm). It is likely, however, that the resolution could be increased through the use of thinner sections, low-electron-dose photography, and image analysis methods applied to the ubiquitous *en face* views present in the sections.

The results of this study clearly establish that in regions of high acetylcholine receptor density, the cytoplasmic membrane surface bears a specialization which projects or lies beyond the surface of the bilayer. Assuming a width of 4 nm for the bilayer, and assuming that the middle lamina in fact corresponds to its center, this specialization extends \sim 4.5 nm beyond the cytoplasmic surface, while the receptor protein extends \sim 4.5 nm beyond the extracellular surface. Thus, the membrane is approximately symmetric with respect to the widths of its two sides. The estimate for the length of the outer, extramembrane portion of the receptor is less than the usually cited 5.5 nm (3, 16). However, it was assumed in the previous measurements that negative stains do not penetrate below the membrane surface. Since the middle lamina in negatively contrasted membranes is only 2 nm wide, that assumption may not be correct. If negative stains and the tannic acid-derived contrasting material do in fact penetrate to a similar degree, the measurements for the outer half of the membrane obtained here and derived from results of conventional negative staining are in close agreement.

These results confirm the symmetric model of the membrane proposed from images of positively contrasted membranes (32). The need for a stringent test of that model arose because the possibility that the inner surface image in positively contrasted membranes could be due to intense poststaining of very little material could not be eliminated. The desired test is particularly well provided by negative contrasting for thin-section electron microscopy, since the image at any point is an average through the section at that point, taken over molecular regions which exclude the contrasting material and intervening regions which are filled with the material. Thus, the strong intensity of the inner surface image shows not only that the molecular elements of the corresponding structure exclude the contrasting material well, but also that they must occur at rather high densities through the thickness of the sections.

Other data obtained in this study, but not presented here, show that the need for this test was not a trivial concern. The plasma membrane of Schwann cells bears a specialization on its outer surface which is thick and very intensely stained in positively contrasted electroplaques and isolated membranes (32). In spite of the very dense appearance of this structure, I have been unable to see it in conventional or tannic acid-mediated negative contrasting of tissue or membranes.

Some of the features of the inner surface image reported here have been recorded previously. Tsuji (43) used negative staining to visualize the postsynaptic membrane in ultrathin cryosections of fixed electric tissue from *T. marmorata*. His most directly transverse view (Fig. 2a of his paper, upper arrow) clearly shows traces of the inner surface bar and the stain-filled region lying just below the middle lamina, as well as the outer surface projections (receptor) which were the main subject of his report.

The symmetric model established here contrasts with two asymmetric models which have been derived from analyses of x-ray scattering by membranes from *T. californica* (16, 27). Agreement is good between the widths of the outer half of the membrane in those models ($l_o = 8$ and 6.5 nm, respectively)

and the width obtained here (~6.5 nm), but not for the inner half ($l_i = 3.4$ and 3.9 nm, respectively, vs. ~6.5 nm) or for l_o/l_i (2.3 and 1.7, respectively, vs. 1.0). That is, in the calculated profiles of average electron density across the membrane, there is no electron density above that of background solvent at points 4–6.5 nm from the membrane center in the cytoplasmic direction. Proteins, even particularly hydrophobic ones, and carbohydrates have electron densities which are greater than those of water or physiological salt solutions (2, 14). Thus, the electron-density profiles and the present images are compatible only if the inner surface image at these points is assumed to arise from amounts of protein which are too small to be detected in the scattering experiments. The intensity of the image makes this unlikely. Since the membranes used almost certainly contained the full complement of receptor and peripheral membrane proteins found in the preparations studied here (8, 16, 27, 37), there seems to be no obvious explanation for the contradictory findings.

The clearest change in the broad image after alkaline extraction is the elimination of the most strongly contrasted feature of its inner half, i.e., the bar or line at its innermost edge. In addition, the intensity of contrasting of the inner surface is generally weakened. Removal of the component or components which give rise to these portions of the image is a plausible explanation for this result. Of the proteins extracted from preparations of postsynaptic membranes in this and other laboratories (8, 22), none of the minor ones are known to be components of the receptor-containing membranes, while a major protein of 90,000 mol wt is clearly associated with contaminating membranes (19, 46). The 43K protein, however, is believed to be a specific component of the postsynaptic membrane (8, 10, 36, 37). In addition, it is not modified by trypsin (44) or lactoperoxidase-catalyzed iodination (29, 30) unless steps are taken to cause entry of the enzymes into the sealed vesicles which the postsynaptic membranes form. And antibodies directed against the 43K protein from *T. californica* bind to the rat neuromuscular junction in a distribution which is essentially identical, at the fluorescence microscope level, to that of the receptor (10). Thus, an obvious interpretation of the results of this study, that the bar or line may arise principally from the 43K protein, is compatible with and supported by considerable independent evidence.

The amount of 43K protein associated with the membrane has not been measured, but the similar staining intensities of the 40,000-dalton (or alpha) subunit of the receptor and of the 43K protein on SDS gels (8, 22, 37) suggest that there may be approximately two copies of 43K protein per receptor molecule (since there are two alpha subunits per molecule [26]). By further assuming square packing for receptors with a 9-nm diameter at their broadest point (3, 4, 47) and a density of 1.33 g/ml for the 43K protein, one can calculate that there is enough 43K protein to form a continuous layer, slightly more than 1 nm thick, under the zone of receptors. Thus, even with the unlikely assumption of a continuous layer, there appears to be enough 43K protein associated with the membrane to account for the bar of contrasted material.

The above analysis provides strong but inconclusive evidence for correlation of the bar with the 43K protein, because it requires the assumption that SDS gels faithfully reveal the nonreceptor protein composition of the membranes. The possibility that a major protein has not been detected because it stains poorly or not at all is unlikely, since essentially identical results are obtained with Coomassie Blue and silver staining

methods (unpublished results). Less easily rejected is the possibility that a major protein may remain associated with the membrane in its appropriate place, while having been proteolyzed during preparation of the membranes to the point that it or its degradation products are not detected on gels. This is the accepted explanation (18) for the commonly observed absence of the γ subunit of the receptor, as in Fig. 6. Extractable proteins in addition to those found here have also been found in some instances (1, 8, 15, 45). The relevance of these observations to the present problem is unclear, however, since the methods of membrane preparation may differ, the purity of the preparations is almost always difficult to judge, and, in one case (15), two major proteins extracted with the 43K protein apparently had no counterparts in the intact membranes. For these reasons, the precise protein composition of the membranes used in this study and, hence, the proposed correlation, remain subject to some uncertainty.

A somewhat different model has been proposed by Changeux and colleagues (3–5, 35) for the membrane from *T. marmorata*. These authors describe the inner lamina of positively contrasted membranes as thin, but adorned with “condensations” of material which they have correlated with the 43K protein (5). Micrographs of selected membranes at high magnification suggest that these condensations take remarkably diverse forms (cf. Figs. 3b and c of reference 5). However, in a recent survey micrograph (Fig. 3a of reference 5), these condensations appear to be of two main types. One consists of irregularly distributed patches which project as much as 40 nm beyond the membrane center. The second is rather uniformly a component of regions of high receptor density, is approximately as wide as the thick outer lamina, frequently has a well-defined inner edge, and contains no substructure (in contrast to the outer lamina). These are the characteristics of the thick inner lamina described for *Narcine* (32). In accord with this similarity, tannic acid-mediated negative contrasting of membranes from *T. marmorata*, kindly supplied by Drs. J.-P. Changeux and A. Sobel, reveals a cytoplasmic surface image which is in every detail similar to those shown here (unpublished results). In a survey of a single section, this image was the predominant one in 75 of 83 postsynaptic membranes which were negatively contrasted on both sides. Projecting condensations could also be seen occasionally although less frequently than in positively contrasted membranes, and they clearly are a new structure identified by these investigators for the isolated membranes. These experiments suggest that, aside from the projecting condensations, the differences between the symmetric and asymmetric-with-condensations models may be due more to technical details of sample preparation and perhaps emphasis in reporting than to major differences in structure of the membranes from different species of ray.

The role of the 43K protein in the postsynaptic membrane is unknown. If its correlation with the bar is correct, the location of the bar would suggest that the 43K protein intermingles with and extends somewhat beyond the cytoplasmic ends of the receptor molecules (see Table I). An interaction between the receptor and the 43K protein can also be inferred from the observations that, after alkaline extraction, the sensitivity of the receptor to trypsin attack (15, 44) and to heat denaturation (31) increases, the receptor becomes rotationally mobile (20, 28), and it may assume a more disordered distribution (1). The receptor also becomes dispersed as individual molecules in the plane of the membrane when alkaline-ex-

tracted, but not intact, membranes are caused to fuse with liposomes (5). These observations lead to the attractive hypothesis (1, 15, 20, 28, 44) that the 43K protein may serve to cross-link or anchor receptor molecules to one another, thereby stabilizing postsynaptic accumulations of receptor. On the other hand, alkaline extraction is a harsh, often long, procedure, and it is not yet clear whether alteration of the lipid portion of the membrane has any effect on these parameters.

Information on the morphology of the alkaline-extractable material in planes parallel to the bilayer of the isolated membranes or of the membrane *in situ* would be useful for analysis of its function. Such information is presumably contained in *en face* views of negatively contrasted membranes such as Fig. 5B, and could possibly be extracted by appropriate image analyses. Visual inspection of numerous examples, both in isolated membranes and in electric tissue, has failed, however, to reveal any systematic structure which might be ascribed to this material.

The 43K protein is currently stimulating considerable interest as the first protein specific to a cholinergic postsynaptic membrane to be discovered by biochemical instead of physiological means. This interest is heightened by the demonstration that a 43K-like protein is probably highly concentrated in the postsynaptic membrane of the rat neuromuscular junction (10). This demonstration made use of frozen sections, so that the antiserum had access to both sides of the membrane. However, if the cytoplasmic localization of the 43K protein proposed here is correct, a structure like the bar should be discernible at the endplate. In results to be presented elsewhere (R. Sealock, submitted for publication), I find that such a structure is clearly evident at endplates in mouse sternomastoid muscles subjected to tannic acid-mediated negative contrasting. One can hope that this apparent discovery of an endplate-specific protein via biochemical study of electric tissue is only the first of several.

I thank Mr. Michael Spillane for excellent technical assistance throughout this project, and Dr. Alan E. Blaurock for a helpful discussion of x-ray diffraction by membranes. Drs. Jean-Pierre Changeux and André Sobel were particularly generous in providing a sample of membranes from *T. marmorata*.

This work was supported by a grant from the National Institutes of Health (NS15293).

Received for publication 8 June 1981, and in revised form 23 September 1981.

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