Visualization of the Cytoplasmic Surface of *Torpedo* Postsynaptic Membranes by Freeze-Etch and Immunoelectron Microscopy

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Abstract. The synapse-specific M_r 43,000 protein (43K protein) and the acetylcholine receptor were visualized by freeze-etch immunoelectron microscopy in preparations of purified *Torpedo* postsynaptic membranes. Vesicles were immobilized on glass and then sheared open by sonication to expose the cytoplasmic surface. Membranes were labeled with monoclonal antibodies to the 43K protein or the acetylcholine receptor. The cytoplasmic surface was devoid of filamentous structure, and the 43K protein and the cytoplasmic projection of the acetylcholine receptor were associated with prominent surface particles. Acetylcholine receptor and 43K protein, in membrane surfaces in direct contact with glass coated with polyornithine, segregated into dense particle aggregates separated by smooth membrane patches, whereas those in contact with glass coated with Alcian Blue underwent little or no detectable rearrangement. After treatment of vesicles at alkaline pH to remove the 43K protein, the cytoplasmic surfaces were still covered by a dense array of particles that were more uniform in shape and appeared slightly shorter than those seen on unextracted membranes, but similar in height to the extracellular projection. Monoclonal antibodies to the acetylcholine receptor labeled these particles, while antibodies to 43K protein did not. We conclude that the 43K protein is in direct association with the receptor and that complexes of the receptor and 43K protein can undergo surface-induced lateral redistribution. In addition, the cytoplasmic projection of the acetylcholine receptor is sufficiently large to be readily detected by freeze-etch electron microscopy and is similar in height to the extracellular projection.

NICOTINIC postsynaptic membranes isolated from *Torpedo* electric organ contain two predominant proteins, the acetylcholine receptor (AChR)[1] and a nonreceptor protein of M_r 43,000 (43K protein), a peripheral protein on the cytoplasmic surface (29, 30, 34, 42, 45). By biochemical and immunological criteria, 43K protein is distinct from actin and creatine kinase, proteins of the electrocyte cytoplasm that are found in some *Torpedo* membrane preparations (2, 18, 34). In the *Torpedo* electrocyte the 43K protein is the only known protein whose distribution is coextensive with AChR (39), and in *Torpedo* electric organ and skeletal muscle, AChR and 43K protein are present in approximately equal concentrations while they are undetectable in other organs (26). Antibodies against *Torpedo* 43K protein recognize determinants highly concentrated in the cytoplasmic side of vertebrate muscle endplate (15, 16), and in *Xenopus* muscle cell cultures 43K protein is concentrated at sites of AChR clusters (32).

Because of this association, 43K protein has been implicated in the clustering or stabilization of clustered AChRs. Accumulated evidence, although indirect, supports this interpretation. (*a*) The 43K protein is in close proximity to AChR, since it can be cross-linked to AChR β-subunit (9). (*b*) The rotational and translational mobilities of membrane-bound AChR are greatly enhanced in membranes depleted of 43K protein by alkaline extraction (27, 37). The mechanism through which 43K protein might contribute to receptor immobilization is unclear. Detergent solubilization of *Torpedo* membranes results in the disruption of any direct interaction between AChR and 43K protein, since the two proteins are readily separated by affinity chromatography or sucrose density centrifugation (13, 40). Purified 43K protein readily associates with liposomes of various compositions, an association disrupted by alkaline pH but not by high salt (35).

Contributing to the uncertainty of how 43K protein may interact with the AChR is the lack of direct structural information about either 43K protein or the cytoplasmic projection of the AChR. The extracellular projection of the AChR has been characterized by deep-etch and negative stain electron microscopy as a ringlike particle 8–9 nm in diameter that projects ~5 nm beyond the bilayer surface (10, 21, 24). Estimates of the cytoplasmic projection of mass vary from 1.5 (23) to 4 nm (8), without information about the relative contributions of AChR and 43K protein. In membranes, 43K proteins are close enough together to interact and form cross-

1. *Abbreviations used in this paper*: AChR, *Torpedo* nicotinic acetylcholine receptor; 43K protein, the major membrane-bound M_r 43,000 protein of *Torpedo* postsynaptic membrane.

linked homopolymers (35), and it has been suggested (11) that 43K protein might exist as a filamentous network analogous to the cytoskeletal structures of the erythrocyte membrane (3). To provide direct information about the structure of 43 K protein and the cytoplasmic projection of the AChR, we have used monoclonal antibodies to 43K protein and AChR in conjunction with freeze-etch electron microscopy under conditions favorable for viewing the cytoplasmic surface of *Torpedo* membranes at a resolution high enough to discern macromolecular structure. A preliminary report of these studies has appeared (7).

Materials and Methods

Isolation of Nicotinic Postsynaptic Membranes

AChR-rich membranes were isolated from the electric organ of *Torpedo californica* by the procedure of Sobel et al. (41), except that fresh electric organ was homogenized in the presence of 10 mg leupeptin, 10 mg pepstatin, 5 mg aprotinin, 18 mg phenylmethylsulfonyl fluoride, 160 mg benzamidine, and 0.02% NaN_3 in 1 liter H_2O. The final membrane suspensions in 38% sucrose-0.02% NaN_3 were frozen in liquid nitrogen and stored at −80°C under argon. Membrane suspensions prepared from five fish bound 1.5–2 μmol ACh per gram protein, as measured by a direct [^{3}H]acetylcholine binding assay (5). For some experiments, membrane suspensions were further purified by velocity sedimentation according to the procedure of Jeng et al. (22), resulting in preparations binding 2.5–3 μmol [^{3}H]acetylcholine per gram protein.

Removal of Peripheral Proteins from AChR-rich Membrane Suspensions

43K protein and other nonreceptor peripheral proteins were removed from isolated AChR-rich membranes by alkaline extraction at pH 11 (29). Membrane suspensions were pelleted by centrifugation and resuspended in water at 4°C (5 mg protein/ml), and the pH was adjusted to pH 11 with 1 N NaOH. After 1 h at pH 11, membranes were pelleted and then resuspended in 36% sucrose-0.02% NaN_3 and then frozen in liquid nitrogen and stored at −80°C until use.

Preparation of Antigens

43K protein and AChR α-subunit were isolated by preparative tube gel electrophoresis using a preparative gel cell (Savant Instruments, Inc., Hicksville, NY). To purify 43K protein, alkaline extract (0.8 mg protein) was subjected to electrophoresis on a 1.4-cm-wide cylindrical gel containing an 8.5-cm separating gel (8% acrylamide) and a 2-cm-long stacking gel (4% acrylamide). Electrophoresis was carried out overnight at 15 mA, and proteins were eluted from the bottom of the gel and collected as 1-ml fractions in 10 mM Tris HCl, pH 8.5, 1 mM dithiothreitol (DTT). Aliquots of each fraction were analyzed by analytical SDS-PAGE, and those fractions containing pure 43K protein were pooled and concentrated by vacuum centrifugation. Typically ~120 μg of pure 43K protein was obtained from 0.8 mg of extract. AChR α-subunit was purified from AChR-rich membranes after alkaline extraction. Receptor subunits were isolated by the same procedure as used for 43K protein, except that proteins were eluted from the gel into 10 mM Tris HCl, pH 8.0, 1 mM DTT, 0.1% SDS. Typically, 250–500 μg of α-subunit was isolated from 12 mg of AChR-rich membranes. Pure 43K and α-subunit were stored until use in small aliquots at −80°C under argon.

Production and Screening of Monoclonal Antibodies

BALB/c mice were immunized intraperitoneally with 35–40 μg of 43K protein emulsified in complete Freund's adjuvant (total vol, 0.4 ml). The injection was repeated 21 and 43 d later using 43K protein in incomplete Freund's adjuvant, and 4 d after the last injection spleens were removed for cell fusion. For AChR α-subunit, mice were injected intraperitoneally initially with 20 μg emulsified in complete Freund's adjuvant. This was followed at 14 d with 20 μg and at 24 d with 50 μg α-subunit (both in incomplete Freund's adjuvant) and at 25 d with another 50 μg (in PBS). Spleens were removed 3 d after the last injection. Spleen cells were fused with SP2/0 myeloma cells, and cells were plated in five 96-well microtiter plates. The hybridization and cloning procedures, which followed standard protocols, were carried out in the Washington University Hybridoma Center.

Hybridoma supernatants were screened initially by ELISA using the protocol of Douillard and Hoffman (12) with modifications. To screen for monoclonals recognizing 43K protein, wells of microtiter plates (Immunoplate 1; Nunc, Roskilde, Denmark) were coated for 12 h at 4°C with 0.15 μg 43K protein (in 0.1 ml carbonate-bicarbonate coupling buffer). For AChR monoclonals, wells were coated with 2.5 μg of SDS-denatured *Torpedo* membranes prepared by treating a membrane suspension (5 mg protein/ml) with 0.25% SDS followed by 200-fold dilution in coupling buffer. Wells were then washed with PBS and left for 1 h at room temperature in a blocking solution of 4% BSA in PBS. Wells were then washed again and incubated for 2 h with 70 μl of hybridoma supernatant. After removal of the supernatants, wells were washed with PBS containing 0.05% Tween 20 and then incubated for 2 h with a 1:1,000 dilution in PBS-Tween of alkaline phosphatase–labeled rabbit anti-mouse IgG and IgM antibodies (Sigma Chemical Co., St. Louis, MO). The wells were washed again before adding substrate (0.1% *p*-nitrophenylphosphate in 0.2 M Tris, 0.5 mM $MgCl_2$, 0.02% NaN_3, pH 9.5). Reaction was terminated by addition of 3 M NaOH. Supernatants from positive wells were then screened further by immunoblotting against AChR-rich membranes resolved by microslab gel electrophoresis (see below). For screening of supernatants, 30 μl of supernatant was diluted to 1 ml for incubation with a strip (3 mm) of the nitrocellulose replica. Cells of interest were expanded and cloned by limiting dilution.

The subclass of monoclonal antibodies was determined by enzyme immunoassay using a double antibody detection system (HyClone Laboratories, Logan, UT). Microtiter plates were coated with goat anti-mouse immunoglobulins. After application of hybridoma supernatants, the wells were exposed to rabbit antisera specific for mouse isotypes and then to goat- antirabbit immunoglobulin coupled to peroxidase.

Ascites fluid was produced by injecting 10^6–10^7 hybridoma cells into BALB/c mice primed with pristane 7–10 d previously. Tumor-bearing mice were killed and the ascites fluid collected. Cells recovered from the ascites fluid by centrifugation were passaged into additional mice, while the supernatants were frozen.

Gel Electrophoresis and Immunoblotting

The polypeptide composition of *Torpedo* membranes was analyzed by SDS-PAGE (25). Optimal resolution of 43K and AChR α-subunit was obtained with a resolving gel containing 8% acrylamide, 0.32% *N,N'*-methylenebis acrylamide, and a stacking gel with 4% and 0.16% crosslinker. Two-dimensional gel electrophoresis was accomplished essentially as described by O'Farrell (31). Sample buffer was composed of 9.95 M urea, 2% NP-40, 100 mM DTT, 0.02% SDS, 0.1% Triton X-100, 2% ampholines (LKB Instruments, Inc., Gaithersburg, MD). Ampholines were composed of three parts pH 3.5–10, one part pH 5–7, and one part pH 7–9. Isoelectric focusing was performed for 16 h.

For immunoblot experiments, membranes were subjected to one- or two-dimensional gel electrophoresis, and then the gel was soaked for 1 h in 25 mM $NaPO_4$, pH 6.5. A replica of the gel was prepared by electrophoretic transfer (2.5 h at 175 mA) of the proteins to nitrocellulose paper. After incubation of the replica in 10 mM $NaPO_4$, pH 7.4, 0.5 M NaCl, 0.05% Tween 20 (blot buffer) for 30 min, it was incubated for 2 h with antibody (diluted appropriately into blot buffer containing 1% BSA), washed three times with blot buffer, and then incubated for at least 1 h with biotinylated anti-mouse IgG antibody (Vector Laboratories, Inc., Burlingame, CA), 1:200 in blot buffer. After another wash, the replica was incubated in Vectastain ABC Reagent, washed three times in PBS, and reacted with 3,3' diaminobenzidine for 30–60 s.

Preparation for Freeze-Etch and Antibody Labeling

For most experiments, membranes were first immobilized on glass disks coated with 0.3% polyornithine (dissolved in distilled water) or 1% Alcian Blue (43). Aliquots of membranes (4 μl) suspended at 2–4 mg protein/ml in 36% sucrose were placed on glass disks (3-mm diam) attached to 35-mm petri dishes to allow easy rinsing and sonication. Membrane suspensions were left on disks for 1–10 min at 23°C.

To expose the cytoplasmic surfaces of receptor-rich membranes, vesicles were opened by osmotic shock followed by sonication. The petri dish containing the disks was rinsed once with distilled water and then placed at the bottom of a beaker containing ~250 ml of distilled water. A probe sonicator with a 1/8-in. tip was then placed directly over a disk (at ~3/4–1 in. distance). Sonication (23 KHz) was for ~4–5 s for each disk at a power output

of 5 W. Unless indicated, membranes were fixed with 2% glutaraldehyde in PBS before freezing.

To label intact or sonicated membranes, a 5-μl aliquot of ascites diluted with PBS to the appropriate concentration was applied directly to membranes (before fixation) attached to the glass. Antibody incubation was 1–2 h followed by several quick rinses with PBS. To prevent evaporation of fluid during the incubation, it was important that the fluid form a distinct drop on the surface of the glass disk. In addition, care had to be exercised during rinses or changes of solution to avoid unintentional drying of the attached membranes. When protein A–colloidal gold (Janssen Life Sciences Products, Piscataway, NJ) was used as a secondary label, it was applied for 30 min at a 1:20 dilution followed by three rinses in PBS.

Electron Microscopy

For rapid freezing, membranes immobilized on glass disks were dipped in distilled water to remove salts, blotted free of excess fluid, and then immediately injected (by a spring-driven device) into a stirred mixture of propane/ethane (~3:1), cooled to −190°C with liquid nitrogen (6). Samples to be freeze-etched were clamped in a standard specimen holder (Balzers, Hudson, NH) that was covered with a specially made aluminum cap to prevent accumulation of hoarfrost during transfer (20). For etching, specimens were placed in a Balzers 400 freeze-etch device on a precooled stage. Because the fluid layer was very thin, it was not necessary (or practical) to knife fracture the sample before etching. Instead, the aluminim cap was knocked off using the knife under high vacuum and the samples, covered with the precooled knife, were directly etched at −97°C usually for 60 min (temperature was calibrated using a thermocouple attached to the surface of the specimen holder). In most replicas a portion of the membranes remained covered with ice while another portion was clearly air dried. Between these two areas, however, a large region was consistently found where ice had been sufficiently thin to allow excellent freezing and complete etching within the 60 min. Too quick an etching rate (at temperatures higher than −97°C) or too long an etching time (much greater than 1 h) caused collapse of vesicles. This was independent of whether the membranes were directly frozen or fixed before freezing. Only preparations that showed no indication of vesicle collapse were used for analysis. The samples were then rotary shadowed at 20° with platinum or platinum alloys followed by a carbon-backing layer (90°). Thickness of the evaporated metal was controlled by a quartz crystal monitor and was kept constant at 1.2 nm. Platinum alloys were used to minimize grain size and were either tantalum/platinum/iridium (19) or rhenium/platinum/iridium. The characteristics of the metal shadowing and resulting replicas will be described in detail elsewhere (Bridgman, P. C., manuscript in preparation). In brief, the alloys resulted in a detectable and reproducible reduction in grain size but had no other discernible effect on specimen shadowing when compared with platinum.

Replicas were floated off the glass in dilute hydrofluoric acid (2.5%) and rinsed three times with distilled water. They were then directly picked up on formar-coated grids (without digestion of membranes) and viewed in a JEM 100 CX operating at 100 kV. Stereo pairs were obtained with tilt angles of ±10°.

Parallax Measurements

The height of protrusions was measured on stereo pairs using a light spot parallax measuring device (Cartographic Engineering Ltd., Landford, England). Heights were computed with the formula (4)

$$Z = \frac{Ph - Px}{2M \sin Q/2},$$

where Z is the height of protrusion normal to the specimen plane, Ph is the mean value for the top of a protrusion, Px is the mean value for the membrane surface at the base of a protrusion, Q is the tilt angle, and M is the magnification.

All measurements were on vesicles that showed portions of both the extracellular and cytoplasmic membrane surface. Prints were at a final magnification of 200,000. Prints upon which measurements were to be made were chosen so that the image contrast was optimally balanced and consistent. All measurements were made by a single observer.

Density Measurements

Particle densities were measured on high magnification (200,000×) prints. All particles within a defined rectangular area were counted (usually 600–700). On both extracellular and cytoplasmic surfaces rectangular areas were selected so that large areas of particle-free membrane that occurred on many vesicles were not included. At least three vesicles from different replicas were used in each category for measurement unless indicated.

Results

Characterization of Membrane Suspensions and Monoclonal Antibodies

To analyze the structure of the cytoplasmic surface of the *Torpedo* nicotinic postsynaptic membrane, we examined AChR-rich membranes at three different stages of purification: after standard purification by equilibrium centrifugation, after subsequent velocity sedimentation, and after alkaline extraction. When membranes are isolated by equilibrium centrifugation, AChR comprises ~25% of protein as judged by [^{3}H]ACh binding. When analyzed by PAGE (Fig. 1, lane *A*), the nonreceptor 43K protein constitutes the predominant, but not the only, nonreceptor polypeptide. When these membranes are subjected to an additional stage of purification by velocity sedimentation (22), the 43K protein copurifies with receptor while other nonreceptor polypeptides are present in reduced amounts (Fig. 1, lane *B*). After alkaline extraction, the 43K protein as well as other nonreceptor polypeptides are recovered quantitatively in the supernatant (Fig. 1, lane *D*), while the resulting membrane suspension is free of 43K protein (Fig. 1, lane *E*).

Purified AChR α-subunit and 43K protein were used as immunogens for the production of monoclonal antibodies. mAb 2316A is representative of three clones isolated from a single fusion that recognizes a protein of M_r 43,000 by immunoblot (Fig. 2, lane *B*). Creatine kinase and actin, common constituents of the electrocyte cytoplasm, are characterized by relative mobilities in SDS-polyacrylamide gels similar to that of 43K protein. Therefore, the specificity of mAb 2316A was also assessed by immunoblot of proteins resolved by two-dimensional gel electrophoresis (Fig. 3). 43K protein is characterized by isoelectric variants (pI 7–8), whereas creatine kinase is characterized by slightly more acidic isoelectric variants (pI 6.5–7), and actin has a pI ~5.5 (15, 18, 34). A sample containing both *Torpedo* postsynaptic membranes and cytosol proteins was resolved by two-dimensional gel electrophoresis and then transferred to

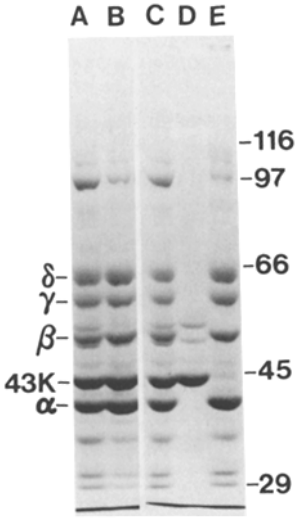


Figure 1. Polypeptide composition of AChR-rich membrane suspensions as determined by SDS-PAGE (Coomassie Blue stain). (*A, C*) Membrane suspensions (containing 1.5 μmol ACh sites per gram protein) isolated by equilibrium centrifugation. (*B*) Membranes after repurification by velocity centrifugation. (*D*) Alkaline extract after treatment of membranes according to the procedure of Neubig et al. (29). (*E*) Membranes after alkaline extraction. The positions of the AChR subunits (α, β, γ, δ) and 43K protein are indicated.

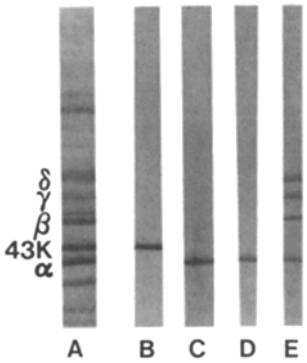


Figure 2. Specificity of monoclonal antibodies as assayed by one-dimensional immunoblot. AChR-rich membranes were subjected to SDS-PAGE (lane *A*, Coomassie Blue stain), and antibody binding was determined after electrophoretic transfer to nitrocellulose by use of a horseradish peroxidase coupled secondary antibody. Lanes *B–E*, mAb 2316A, mAb 2578A, mAb 2578B, mAb 2579B. Ascites fluid diluted 1:1,000.

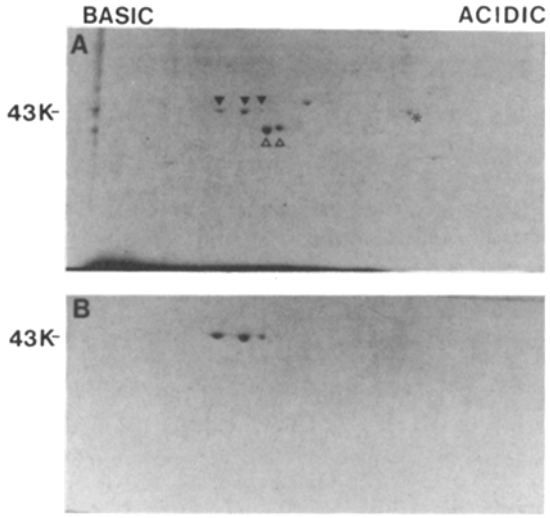


Figure 3. Two-dimensional immunoblot of anti-43K mAb 2316A. A sample containing electric organ cytosol combined with AChR-rich membranes was subjected to two-dimensional gel electrophoresis, and an immunoblot was prepared after electrophoretic transfer of the proteins to nitrocellulose. (*A*) Coomassie Blue stain of the portion of the gel containing immunoreactivity. Solid triangles indicate 43K protein; open triangles, creatine kinase; and asterisk, actin. (*B*) Blot with mAb 2316A, culture supernatant at 1:20 dilution.

nitrocellulose for immunoblotting. As judged by Coomassie Blue stain (Fig. 3 *A*), the sample contained 43K protein as well as creatine kinase and actin, with the identity of creatine kinase and actin confirmed by immunoblot with commercial antisera (data not shown). mAb 2316A recognized the isoelectric variants characteristic of 43K protein (Fig. 3 *B*). In the *Torpedo* postsynaptic membrane preparations used for electron microscopy, creatine kinase was not detectable by immunoblot, whereas actin was identified as a minor component characterized in one-dimensional gels by M_r ~47,000.

From two fusions where AChR α-subunit was used as immunogen, 14 monoclonal antibodies were isolated that recognized AChR α-subunit by immunoblot. The full characterization of these antibodies will be presented elsewhere, but mAb 2578A and 2578B recognized only α-subunit (Fig. 2, lanes *C* and *D*). These antibodies were identified in a screen for antibodies recognizing antigenic determinants exposed on the AChR cytoplasmic surface. By ELISA they bound to cholate-solubilized AChRs, but not to intact membranes, and by immunofluorescence using frozen sections of *Torpedo* electric tissue they recognized determinants restricted to the electrocyte innervated surface (data not shown). mAb 2759A, which recognized an epitope common to all receptor subunits (Fig. 2, lane *E*), also bound to an exposed determinant as judged by immunofluorescence.

Visualization of AChR-rich Membranes

Immobilization of vesicles on glass before freezing provided a particularly good means of providing broad views of both extracellular and cytoplasmic membrane surfaces. This was because the glass surface provided support to vesicles that otherwise collapse during deep etching.

Replicas of membrane suspensions prepared by equilibrium sedimentation contained a population of vesicles (ranging from 0.2 to 1.5 μm) and an occasional membrane sheet. Membrane preparations were usually contaminated with a small number of collagen fibers and filamentous material reminiscent of basal lamina meshworks. The majority of vesicles had an outer surface covered with a dense mosaic of particles or protrusions (8,900 ± 500/μm²; mean ± SEM) that had the characteristic size (7–8 nm) and shape of acetylcholine receptors (Fig. 4) (21). Large AChR containing vesicles tended to flatten against the surface of the glass, resulting in vesicles as that of Fig. 4 that appeared to be "hat"-shaped.

To examine the cytoplasmic surface of membranes, vesicles were opened by osmotic shock and sonication before freezing. The inner or cytoplasmic surface of vesicles contained prominent protrusions arranged in densely packed clusters (Fig. 5). Identical structures were also seen on cytoplasmic membrane surfaces if (*a*) sonication was avoided and vesicles were opened by osmotic shock alone, or (*b*) vesicles in suspension were frozen in PBS, fractured, and briefly (3 min) etched (data not shown). The cytoplasmic surface was not associated with filamentous material under any of the conditions tested. Rarely one or two filaments could be seen on the cytoplasmic surface of a vesicle.

Membranes that had been further purified by velocity sedimentation contained a population of vesicles that were generally smaller and more uniform in size (Fig. 6 *A*). Very little contaminating filamentous material was seen in these preparations. Almost all vesicles were covered with characteristic AChR protrusions. When opened by osmotic shock and sonication (Fig. 6 *B*), the cytoplasmic surface was covered with protrusions identical to those seen in vesicles purified solely by equilibrium sedimentation. Because small vesicles were more difficult to shear open, and when opened revealed a relatively small area of cytoplasmic membrane surface, AChR-rich membranes purified only by equilibrium sedimentation were used in most experiments.

While the particles observed on the extracellular surface were similar in size and shape to extracellular projections of the AChR characterized previously by freeze-etch or negative stain (10, 21, 24), the substantial cytoplasmic structure was novel and surprising. We wished to design experiments to determine whether the observed cytoplasmic structure might be generated as a consequence of the immobilization technique used. This was a concern because cytoplasmic projections were visualized for a portion of the vesicle with the extracellular surface in direct contact with the glass. In

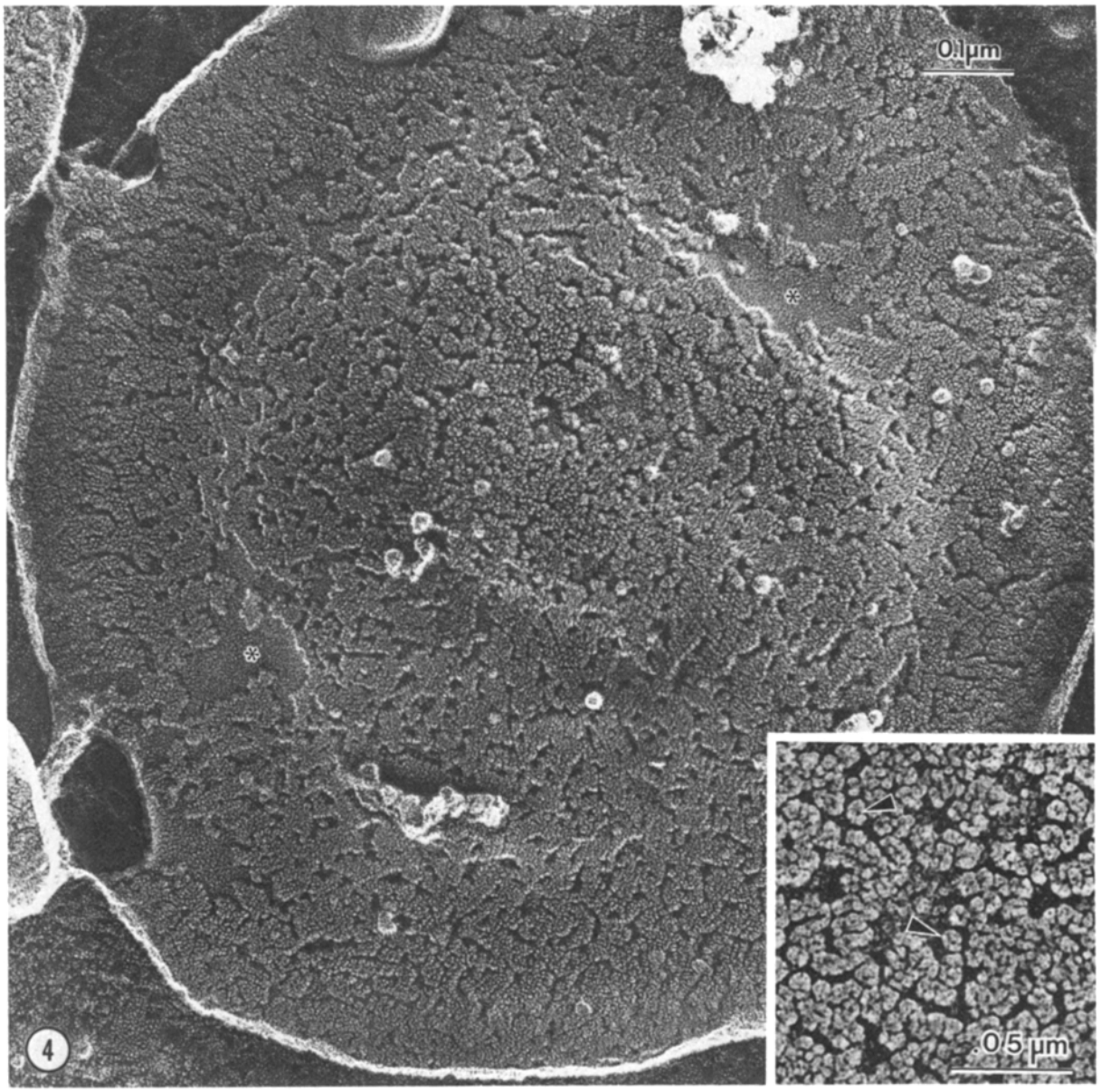


Figure 4. A freeze-etch view of a large vesicle from an unsonicated, directly frozen preparation of *Torpedo* AChR-rich membranes. The vesicle has flattened against the polyornithine-coated glass surface to form a "hat" shape. The vesicle is covered with dense aggregates of surface protrusions, although an occasional area of smooth membrane can be seen (*asterisks*). Individual AChR protrusions are difficult to see at this magnification because of the dense packing and complex geometry of the vesicle. Shadowing was with tantalum/platinum/iridium. (*Inset*) High magnification of individual AChRs which in favorable orientations appear doughnut-shaped (*arrowheads*). From a different vesicle shadowed with rhenium/platinum/iridium.

contrast, extracellular projections were visualized on a different part of the vesicle for which the cytoplasmic projection would be in contact with the ice inside the vesicle. In fact, as can be seen in Fig. 5, the distribution of protrusions on the cytoplasmic surface (dense aggregates separated by patches of bare membrane) did not correspond closely to the more uniform distribution on the extracellular surface.

As one control, membranes were fixed in suspension and compared with those fixed 1 or 10 min after application to the coated glass. Prefixed vesicles were difficult to break open with sonication, but usually a small percentage could be found that did show adequate views of the cytoplasmic surface (Fig. 7 *A*). Protrusions were identical to those seen on vesicles fixed after contacting the polyornithine-coated surface but were distributed more uniformly and corresponded closely to the distribution on the extracellular surface. Vesicles fixed 1 min (Fig. 7 *B*) or 10 min (Fig. 7 *C*) after application to polyornithine-coated glass showed progressing degrees of disparity from the distribution seen on extracellular surfaces. Protrusions were separated from areas of smooth membrane into tightly packed aggregates. Longer times on glass sometimes led to even tighter packing and a netlike distribution of protrusions on the membrane surface.

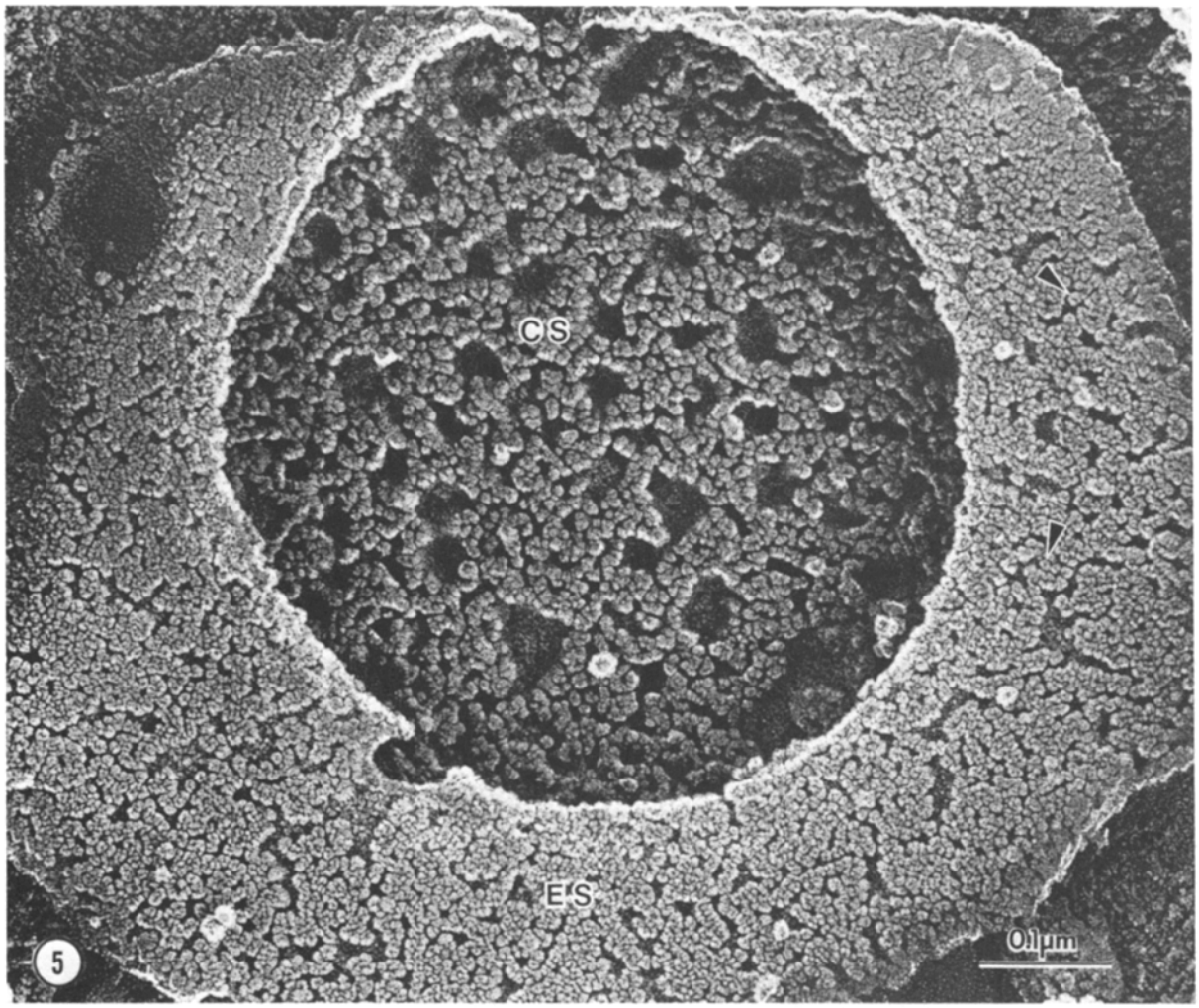


Figure 5. A freeze-etch view of an AChR-rich vesicle that has been sheared open by sonication to reveal its interior. The outer surface of the vesicle corresponds to the extracellular membrane surface (*ES*) and is covered by aggregates of doughnut-shaped protrusions that represent individual AChRs (*arrowheads*). The interior of the vesicle corresponds to the cytoplasmic membrane surface (*CS*) and is also covered with dense aggregates of protrusions. Membranes in this preparation were in contact with the polyornithine coated glass for 10 min before sonication and then fixation with glutaraldehyde. Shadowing was with rhenium/platinum/iridium.

As an additional control, polyornithine was replaced by Alcian Blue (43) for membrane attachment to glass. Alcian Blue is much less polar and charged than polyornithine, and unlike polyornithine, Alcian Blue does not cause segregation of membrane proteins when used to attach reticulocytes to mica (Dr. John Heuser, personal communication). When vesicles were attached to glass using Alcian Blue and then sonicated to reveal the cytoplasmic membrane surface, prominent cytoplasmic projections were still observed (Fig. 8), but there was a clear difference in the distribution of protrusions when compared with vesicles attached using polyornithine. The cytoplasmic protrusions did not segregate even after long periods of contact (1 h) with the Alcian Blue–coated glass. The density of protrusions on cytoplasmic surfaces was 6,400 ± 700/μm². This value is slightly lower than that obtained for the density of extracellular protrusions (8,900 ± 500 μm²). Probably the discrepancy in the two values results from the difficulty in distinguishing individual protrusions in some areas on cytoplasmic surfaces adsorbed to the Alcian Blue.

To control for decoration effects during replication that could influence the appearance of surface particles (1, 46), some samples were rotary shadowed at 90° with a thin layer of carbon (5–10 Å calculated from quartz crystal monitor readings) before shadowing with platinum alloys (44, 46). Thin layers (5 Å) had no detectable effect on the appearance of either extracellular or cytoplasmic particles (Fig. 9). Thicker layers of carbon (10 Å) caused both extracellular and cytoplasmic particles to appear less distinct (not shown, but see Fig. 15 *B*), but did not influence the overall relative appearance of mass.

Antibody Labeling

To determine if the 43K protein contributed to the protrusions associated with the cytoplasmic surface of the membranes, sonicated vesicles were incubated with a monoclonal antibody to 43K protein (mAb 2136A). This resulted in decoration of protrusions with distinct 10–15-nm particles, but only on cytoplasmic membrane surfaces (Fig. 10). Protru-

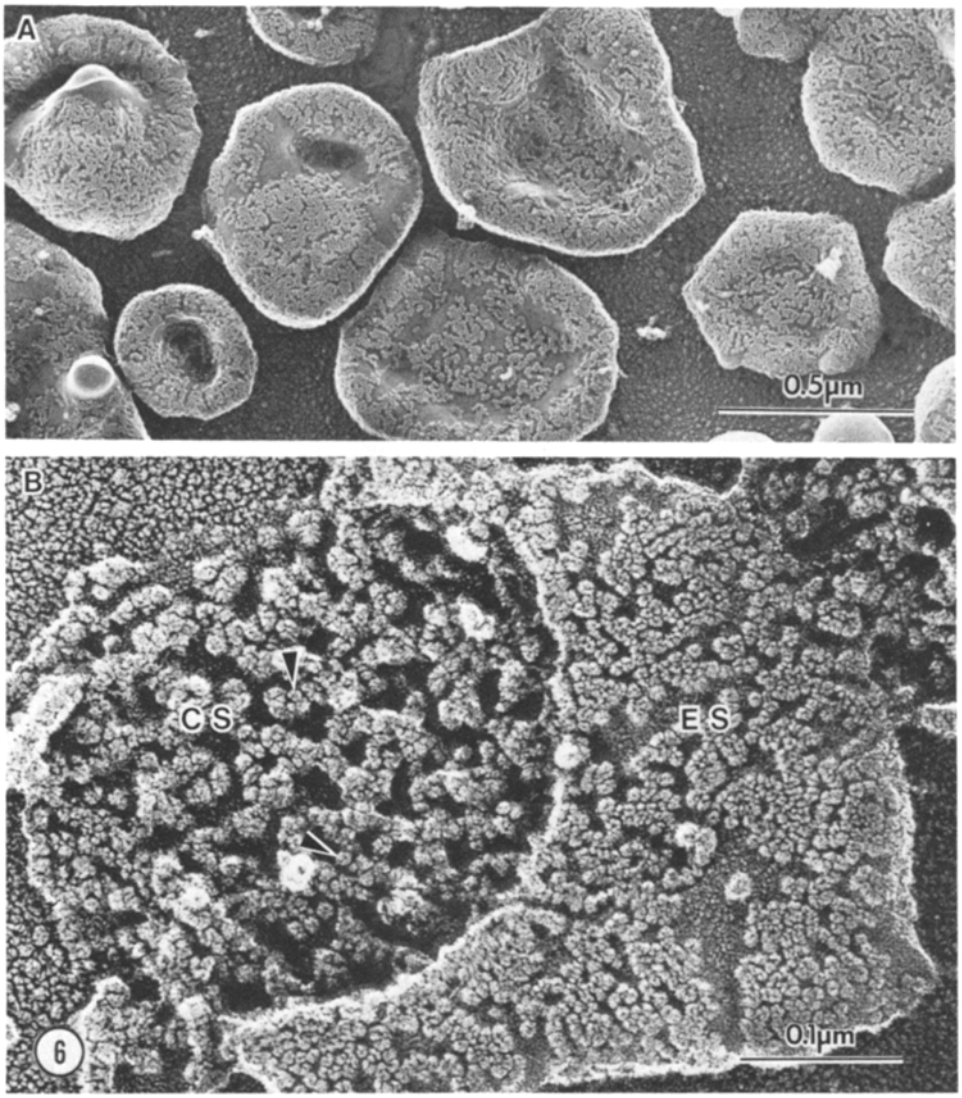


Figure 6. (*A*)A survey view of vesicles from a preparation of *Torpedo* AChR-rich membranes repurified by velocity sedimentation. Vesicles are generally smaller and more uniform in size than those purified by equilibrium sedimentation. (*B*) A vesicle from a velocity purified preparation that has been sheared open by sonication. The cytoplasmic surface (*CS*) is covered with large protrusions (*arrowheads*) which appear identical to those seen in vesicles isolated by equilibrium sedimentation. *ES*, extracellular surface.

sions on the extracellular membrane surface or protrusion-free membrane were not decorated. To be sure that the decorating particles represented antibodies, protein A–colloidal gold was used as a secondary label. Although the density of protein A–colloidal gold labeling was somewhat less than the density of particles in membranes incubated with antibody alone, the distribution was the same (Fig. 11). The lower density of protein A–colloidal gold label probably results from dissociation of some of the antibody during the protein A–colloidal gold incubation, since prolonged wash times also decreased decorating antibody density.

The particles on the cytoplasmic surface appeared heterogeneous in height and shape compared with the extracellular surface. In addition, even high concentrations of mAb 2316A failed to decorate the majority of cytoplasmic protrusions. This could mean that only a portion of the protrusions represent 43K protein, while the others may represent the cytoplasmic projection of the AChR or perhaps other protein(s). To test the possibility that some of the protrusions represent the cytoplasmic projection of the AChR, sonicated membranes were incubated with two monoclonal antibodies directed against a cytoplasmic portion of the AChR α-subunit (mAbs 2578A and 2578B), followed by protein A–colloidal gold. With both antibodies, membranes were labeled only on cytoplasmic surfaces and edges of broken membranes. Label was confined to areas containing protrusions, supporting the idea that some of the cytoplasmic protrusions represent the intracellular projection of the AChR (Fig. 12). While these two monoclonals labeled selectively the cytoplasmic surface of the vesicles, in additional experiments we examined vesicles after incubation with a monoclonal (mAb 139a) that recognizes an extracellular portion of the AChR α-subunit (17). This antibody labeled only extracellular projections, and at the concentrations used (0.4 mg/ml), the density of labeling was similar to that observed for the antibodies labeling the cytoplasmic surface (data not shown).

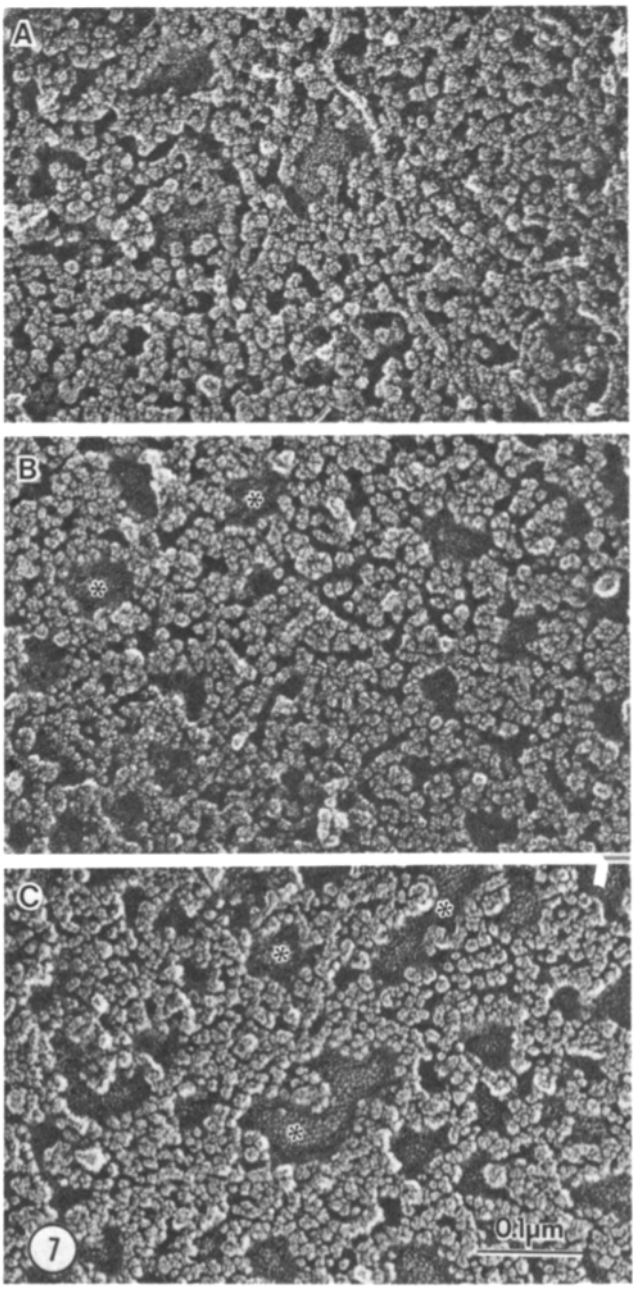


Figure 7. Cytoplasmic surfaces of AChR-rich vesicles attached to polyornithine-coated glass and then sheared open by sonication. Protrusions show rearrangement during prolonged contact with the polyornithine coated surface. (*A*) From a preparation fixed with glutaraldehyde before attachment. (*B*) A preparation that was fixed 1 min after attachment. Small patches of protrusion free membrane (*asterisk*) are more frequent. (*C*) A preparation that was fixed 10 min after attachment. Patches of protrusion free membrane are generally larger and more frequent than in *A* and *B*. Shadowing was with rhenium/platinum/iridium.

Alkaline-extracted Membranes

To determine which protrusions may represent 43K protein and which may represent the AChR, membranes were subjected to alkaline extraction at pH 11 before immobilization on glass. Alkaline extraction removed most of the 43K protein from membranes as shown by SDS-gel electrophoresis (Fig. 1). Membranes that had been subjected to alkaline extraction differed from unextracted membranes in several ways. Large, round vesicles were rare while medium to small irregularly shaped vesicles were more numerous (Fig. 13). The packing and organization of protrusions on the extracellular membrane surface usually differed from that seen on unextracted vesicles. While unextracted vesicles were covered with a fairly uniform, densley packed array of protrusions (Fig. 13 *A*), aggregations of protrusions on alkaline-extracted membrane attached to glass using polyornithine were most often confined to edges of vesicles that were usually flattened against the glass (Fig. 13 *B*). Sometimes aggregates were also seen on small domes rising from the membrane surface away from the glass (Fig. 13 *B*). The remaining portion of the membrane surface away from the glass usually contained loosely packed protrusions often appearing as singlets or doublets.

When alkaline-extracted vesicles were sheared open by sonication to reveal the cytoplasmic membrane surface, they still contained distinct protrusions (Fig. 14). When vesicles were attached using polyornithine (Fig. 14 *A*), these protrusions were usually tightly aggregated and there were also large patches of protrusion-free membrane. When vesicles were attached to glass using Alcian Blue (Fig. 14 *B*), the distribution of extracellular protrusions was more uniform than those on vesicles attached using polyornithine. In addition, the distribution of cytoplasmic protrusions closely resembles that seen on the extracellular surface. The density of cytoplasmic protrusions under these conditions was 6,500 ± 600/μm² (from three vesicles), the same as the density of cytoplasmic protrusons on "native" membranes.

To further control for factors that might produce artifactually prominant cytoplasmic projections, two additional controls were considered. First, to ensure that adsorption to the glass surface had not dramatically affected the cytoplasmic projections, we searched specifically for vesicles that were lying on top of either other vesicles or sheets of membrane. Several examples were found. These showed no detectable difference in the appearance of cytoplasmic particles (Fig. 15 *A*) when compared with those in contact with the glass surface. Second, to ensure that decoration effects were not greatly influencing the appearance of the cytoplasmic particles, a preparation was preshadowed with carbon (10 Å) at 90° before rotary shadowing with platinum alloys (Fig. 15 *B*) (44, 46). Both extracellular and cytoplasmic particles were less distinct but the relative appearances and sizes did not change.

To see if the protrusions remaining on the cytoplasmic surface after alkaline extraction might represent the cytoplasmic projection of the AChR, extracted vesicles were incubated with the monoclonals to the cytoplasmic portion of the AChR. mAbs 2578a and 2578b both labeled cytoplasmic membrane protrusions but were absent from protrusion-free membrane or the extracellular membrane surface (data not shown). In contrast, mAb 2316A recognizing the 43K protein no longer labeled the cytoplasmic membrane surface (and was also absent from extracellular surfaces or protrusion free membranes).

Projected Structure

In stereo views of native membranes, the heights of the cytoplasmic protrusions appeared similar to those on the ex-

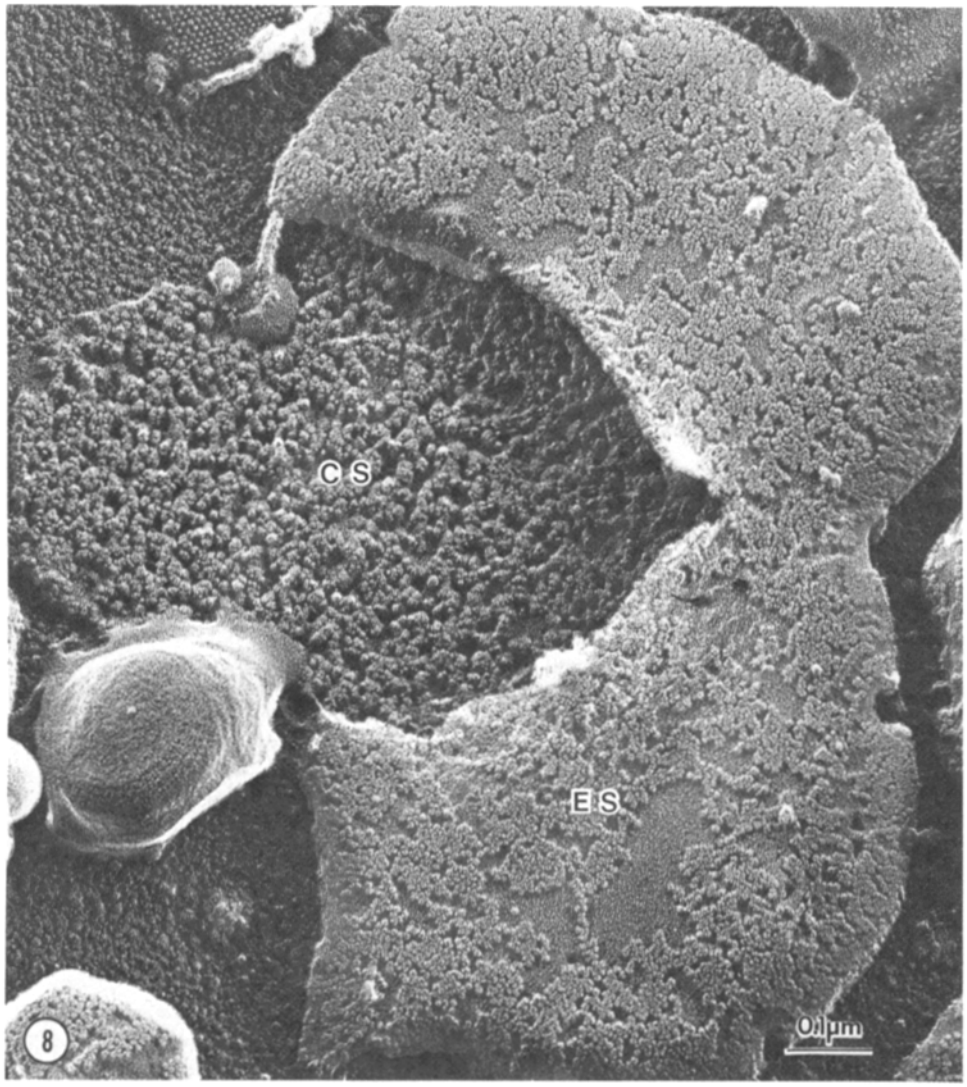


Figure 8. An AChR-rich vesicle from a preparation that was attached to glass using Alcian Blue. Sonication was 10 min after attachment. No protrusion-free membrane areas are present on the cytoplasmic surface (*CS*), suggesting that little lateral redistribution has occurred. Shadowing was with rhenium/platinum/iridium.

tracellular surface, whereas the relative height of the cytoplasmic protrusions appeared reduced on alkaline extracted membranes (Fig. 16). To attempt to quantify these impressions, the projection heights of the protrusions were determined using parallax measurements. In making these measurements several factors were considered. (*a*) The absolute values of the particle dimensions could vary from one preparation to another depending upon the specific shadowing conditions. In addition it would be difficult to calibrate the parallax measurements to determine absolute heights. However, the ratio of extracellular to cytoplasmic heights on a given vesicle should provide an internally consistent measure. (*b*) The dense packing of protrusions made it necessary to restrict height measurements to those protrusions adjacent to patches of smooth membrane large enough to have received platinum shadowing (asterisks in Fig. 16). This provided a means of determining the base of a protrusion. When vesicles were attached to glass coated with polyornithine, large protrusion-free membrane patches (as in Fig. 10) were often pulled down or collapsed below the actual base height of the protrusions. This led on first glance to a mistaken impression that protrusions on the cytoplasmic surfaces were actually much higher than they were. By selecting areas where the smooth membrane was apparently not distorted, measurements could be made on cytoplasmic projections for vesicles immobilized with polyornithine. In addition, measurements were made on membranes attached using Alcian Blue, which seemed to have less effect on the smooth membrane.

In measurements made on four membrane preparations (Table I) the ratio of extracellular to cytoplasmic heights was 0.79 ± 0.09 (range 0.68–0.85) for normal membranes, and that ratio increased to 1.0 ± 0.07 (range 0.93–1.07) for alkaline-extracted membranes. Similar results were obtained on vesicles adsorbed on Alcian Blue–coated glass as on vesicles adsorbed on glass coated with polyornithine. The effect of alkaline extraction was to reduce by ~20% the cytoplasmic projection height without alteration of the extracellular projection height.

Discussion

As observed by freeze-etch electron microscopy, both surfaces of *Torpedo* postsynaptic membranes immobilized on glass were covered with a dense population of particles. On the outer or extracellular surface of membranes, in favorable

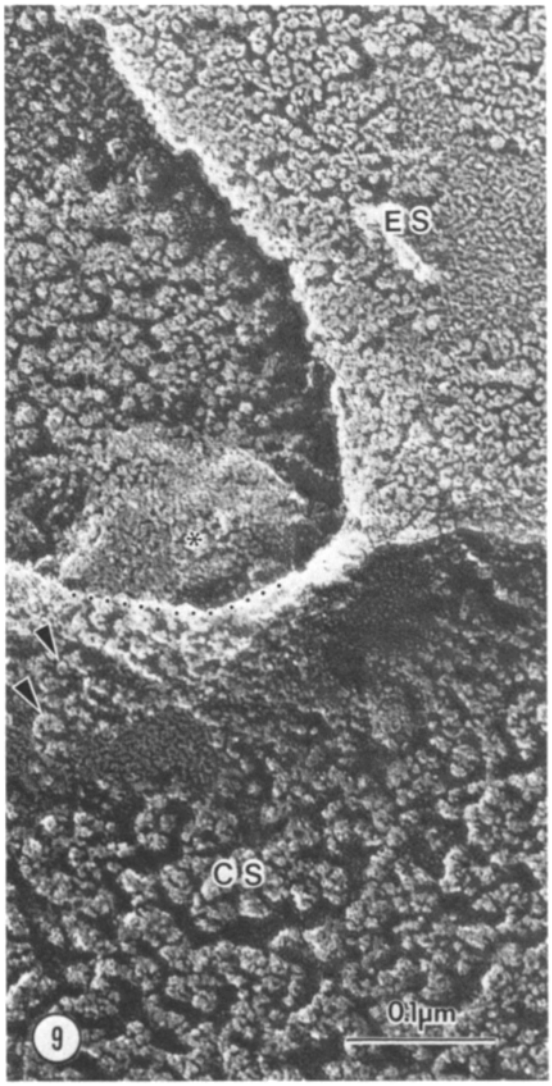


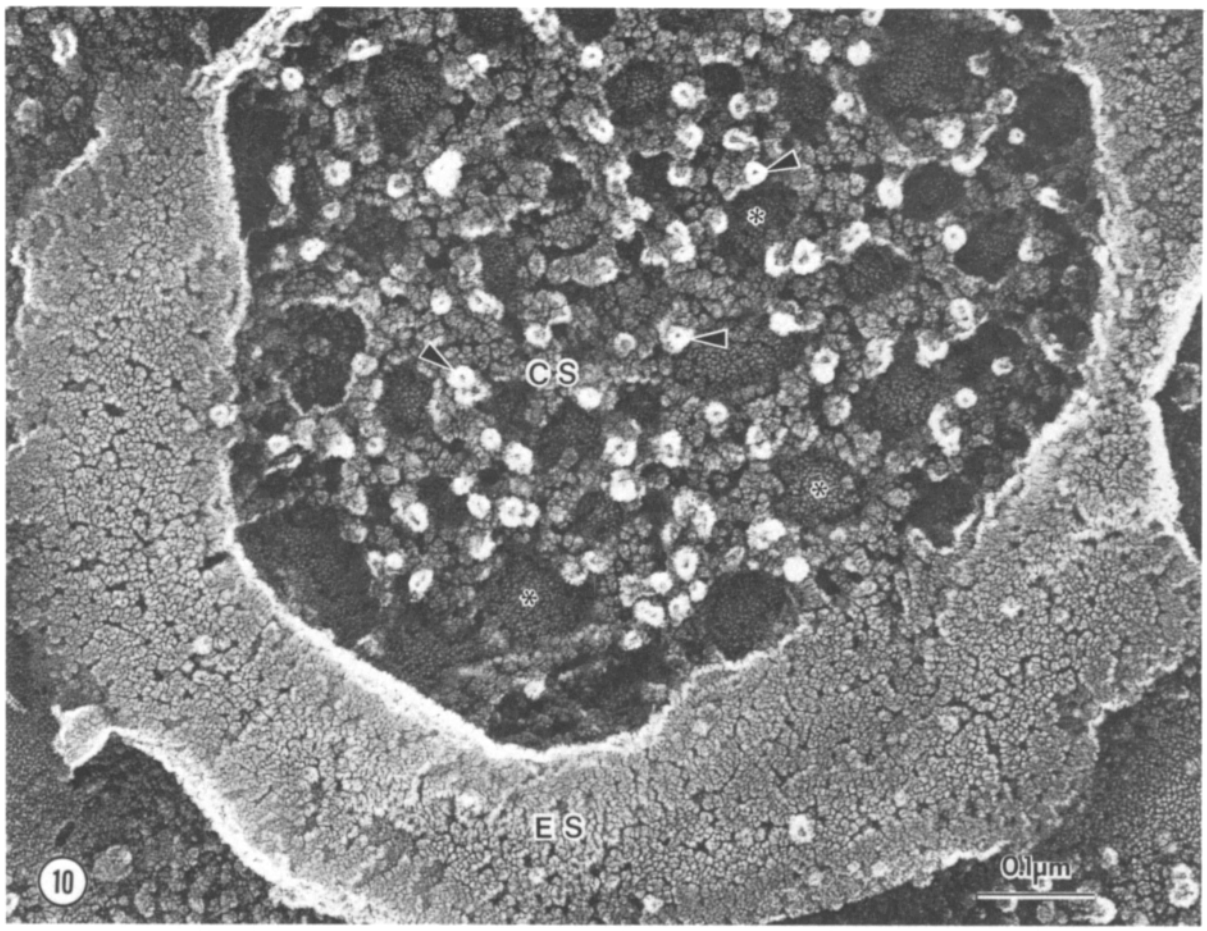
Figure 9. A control for decoration effects on the appearance of surface particles. An AChR-rich vesicle attached to polyornithine-coated glass was (rotary) preshadowed with 4–5 Å of carbon before rotary shadowing with rhenium/platinum/iridium. The thickness of the carbon layer was calculated from quartz crystal monitor readings. A portion of both the extracellular surface (*ES*) and cytoplasmic surface (*CS*) is shown. There is no apparent difference in the relative appearance of the particles on either surface when compared with each other or to particles on vesicles that have not been preshadowed with a thin layer of carbon (compare to Figs. 5 and 8). The lack of any effect on the appearance of particles presumably results from the fact that the carbon layer was so thin. Thicker layers of carbon did change the appearance of particles on both sides of the membrane (see Fig. 15 *B*). It is also worth noting that the edge (*dots*) of the cytoplasmic surface is not in contact with the glass. This is because the edge is resting against a portion of another vesicle (*), which separates the membrane from the glass surface. Although the edge is seen at an oblique angle, particles (*arrowheads*) can be seen.

orientations, the particles appear as the small doughnut-shaped structures characteristic of AChRs seen in previous studies (10, 21). The cytoplasmic membrane surface, which has not been previously observed under conditions that allowed detailed analysis of structure, is also covered with a dense population of particles. Based upon the binding of monoclonal antibodies, we conclude that both AChR and 43K protein contribute to the observed cytoplasmic projections. Although filamentous structures project towards the innervated surface of the *Torpedo* electrocyte (21), no filaments remain in association with the isolated postsynaptic membranes, and 43K protein itself is not organized as a filamentous structure. Under the conditions used for antibody labeling, monoclonal antibodies directed against AChR or 43K protein bound to only a fraction of the cytoplasmic particles. While it is likely that AChR contributes to all the observed cytoplasmic projections, it is not possible to conclude whether 43K protein does so also.

We do not believe that the observed cytoplasmic projections result from a reorganization of membrane structure as a consequence of the adsorption of the vesicles to glass for the following reasons. First, vesicles fixed before adsorption to glass also show identical structures. Second, similar structures are observed for vesicles adsorbed to glass coated with polyornithine and with Alcian Blue, and the latter surface induces no detectable lateral redistribution. Finally, in some cases (as in Fig. 15 *A*), vesicles are not in direct contact with glass but are lying on top of other vesicles, and in these instances the same cytoplasmic structures are observed.

While interactions with the glass surface are one possible source of induced reorganization, a second factor is the possibility that the observed cytoplasmic structures result artifactually as a consequence of the etching and shadowing technique. However, the prominent cytoplasmic projections were seen in both fixed and unfixed samples, with etching times varying from 3 min to 1 h, and in the presence or absence of salt (all samples shown in figures were rinsed in distilled water before freezing because salt deposits can affect the appearance of structure; 28). Furthermore, in each case the extracellular projections remained doughnut-shaped, acting as an internal control. Rotary shadowing can also affect the appearance of structure since the coating procedure has been shown to give an imperfect depiction of surface re-

Figures 10 and 11. (Fig. 10) A view of a sheared open vesicle from a preparation that was incubated for 1 h with a monoclonal antibody to 43K protein (mAb 2316A). AChR-rich vesicles were attached to glass with polyornithine and then sheared open by sonication before incubation with antibodies (ascites diluted 1:5). The white particles, with a dark center (*arrowheads*), are presumed to represent bound IgG. This appearance probably results from the low angle (20°) at which the metal is shadowed. The antibodies project a relatively large distance above the particle to which they are bound and thus receive more metal on their sides (white outer portion) than their top (dark center). Only the cytoplasmic membrane surface (*CS*) is labeled. The label is confined to regions containing aggregated protrusions and is absent from protrusion free membrane (*). From a preparation that was directly frozen without prefixation with glutaraldehyde. Shadowing was with tantalum/platinum/iridium. *ES*, extracellular surface. (Fig. 11) From a similar preparation to that shown in Fig. 10 except the incubation with mAb 2316A was followed by labeling with 10 nm protein A–colloidal gold (*arrowheads*). The distribution and specificity of label is identical to that seen in preparations incubated with the antibody alone. Shadowing was with rhenium/platinum/iridium. *ES*, extracellular surface. *CS*, cytoplasmic surface.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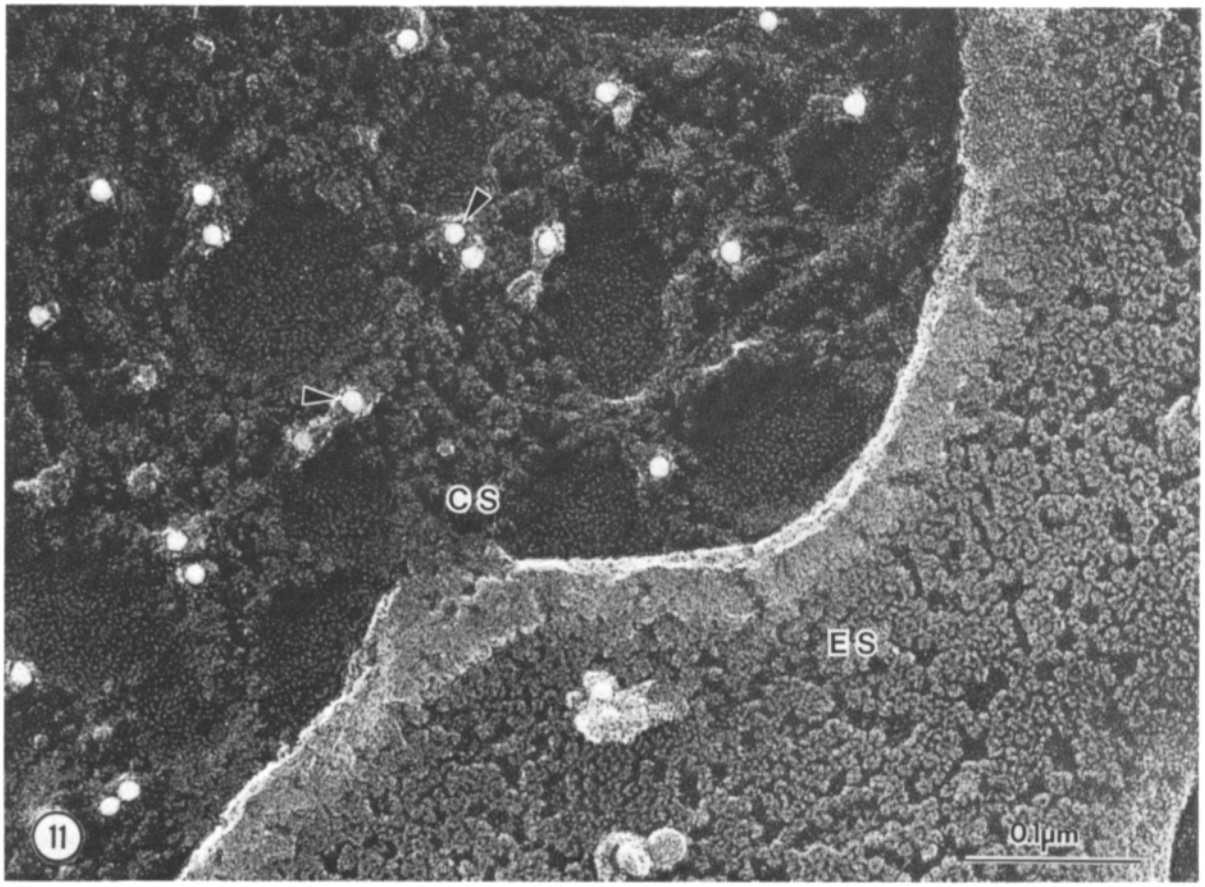
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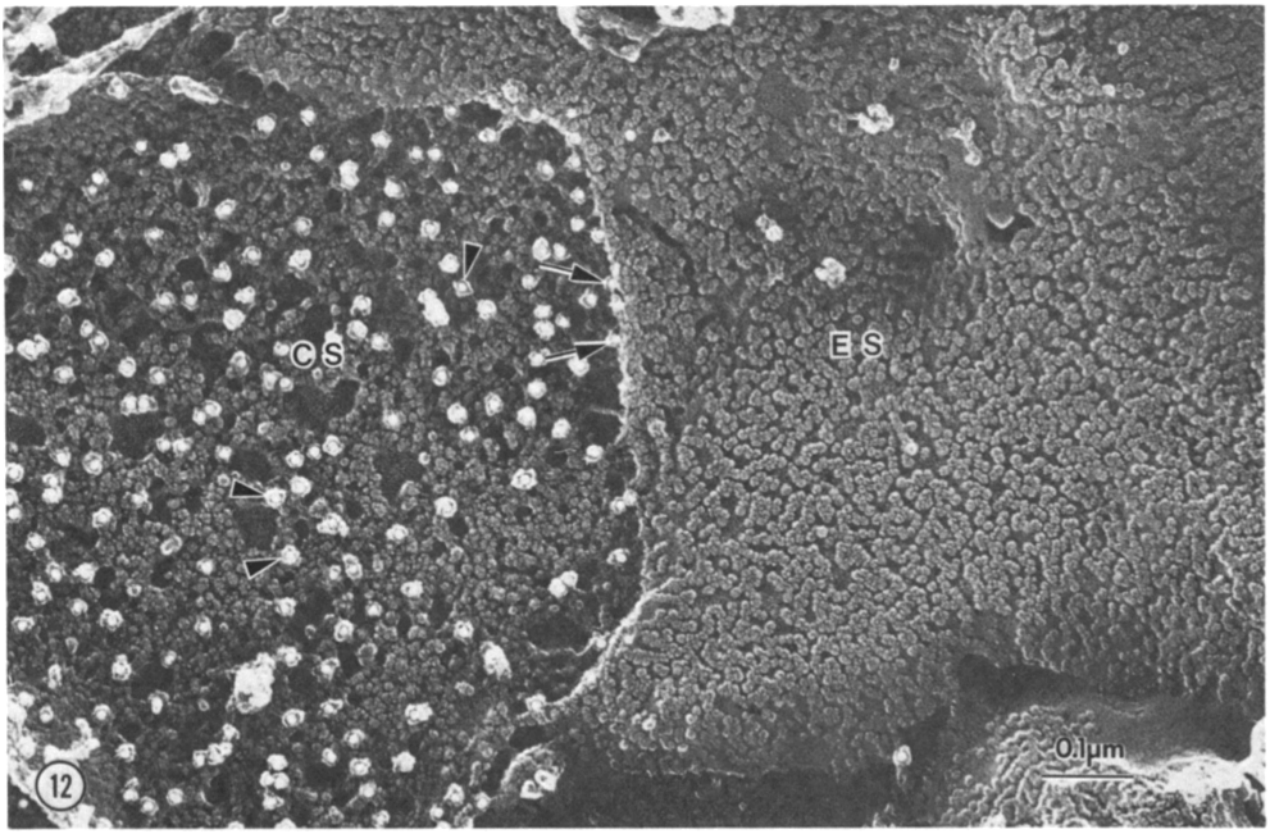

Figure 12. The cytoplasmic surface (*CS*) is shown of a vesicle from an AChR-rich membrane preparation that was sonicated and then incubated with a monoclonal antibody to the α-subunit of the AChR (mAb 2578A 1:5 dilution of ascites) followed by protein A–colloidal gold. Label is confined to areas of aggregated cytoplasmic protrusions (*arrowheads*) and is absent from protrusions on extracellular surfaces (*ES*) or protrusion free membrane. Some label is also seen along the edge of the broken membrane (*arrows*). Shadowing was with rhenium/platinum/iridium.

lief. This is because of decoration effects that arise from the preferential accumulation of platinum grains at certain sites on protein molecules (1, 46). To see if this could significantly affect the appearance of cytoplasmic particles we used a carbon preshadowing procedure, which has been shown to be a good control for decoration effects (44, 46). Results from this procedure showed that decoration has little or no effect on the relative size of the projection made by cytoplasmic particles.

Our results lead us to conclude that there is a relatively large amount of mass associated with the projections on the cytoplasmic surface of the *Torpedo* postsynaptic membrane, a conclusion at variance with several previous studies. While a study of tannic acid–fixed membranes indicated an approximately symmetrical distribution (38), a general model of *Torpedo* membranes based upon combined results from x-ray and negative stain microscopy (23, 24) indicated an overall length of 11 nm with an extracellular projection of 5 nm and a cytoplasmic projection less than 1.5 nm. Recent electron image analysis of the projection structures of frozen suspensions of tubular crystals indicate an overall length of 14 nm with a cytoplasmic projection (3.5–4.5 nm) ~50% that of the extracellular projection (7 nm) (8). While the explanation for these discrepancies is uncertain, several comments are appropriate. (*a*). In the latter study there was uncertainty in the location of the lipid bilayer, which was determined from correlations with negatively stained preparations. (*b*) Only a small portion (several percent) of *Torpedo* membranes spontaneously form tubular crystals after several weeks of incubation. Although 43K protein is presumed to be present in these structures, this has not in fact been directly demonstrated. (*c*) In our images the observed cytoplasmic structures are more heterogeneous in height and shape than those on the extracellular surface. Such a lower degree of order could result in an underestimate of mass in diffraction studies.

After alkaline extraction the AChR is essentially the only protein remaining in the *Torpedo* membranes, but the cytoplasmic surfaces of these membranes still contain particles at the same density as "native" membranes and the particles project a substantial distance from the membrane surface. Monoclonal antibodies to the AChR label the cytoplasmic projections of alkaline-extracted membranes, while monoclonals to 43K protein do not. This suggests that these cytoplasmic protrusions represent primarily the cytoplasmic projection of the AChR. We attempted to quantify by parallax measurements the relative projection heights of the extracellular and cytoplasmic particles. Although these measurements confirm our subjective impression of the relative contributions of mass by the particles on the different sur-

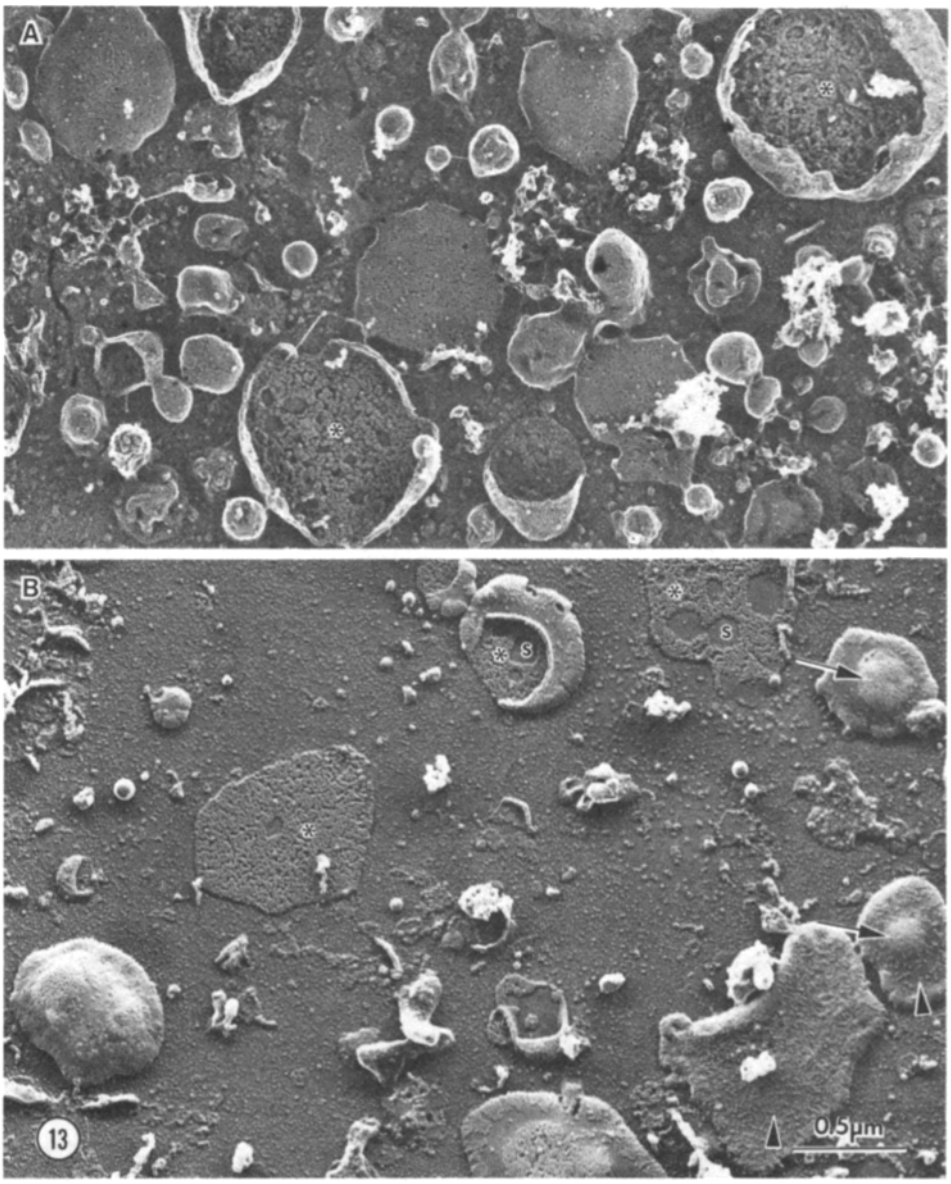

Figure 13. A low magnification survey view of *Torpedo* AChR-rich membranes isolated by equilibrium sedimentation before (*A*) and after (*B*) alkaline extraction. Vesicles were attached to glass with polyornithine and then subjected to sonication. Some remain intact while others have been sheared open (*) by the sonication. After alkaline extraction, protrusions on extracellular membrane surfaces that are not in contact with the polyornithine are either loosely scattered (*arrowheads*) or aggregated along the flattened edges of vesicles or on small domes rising from the central portion of the vesicle (*arrows*). On cytoplasmic membrane surfaces against the glass (*) protrusions are still apparent and are tightly aggregated. Irregular shaped, smooth patches of membrane (*s*) sometimes separate regions of tightly aggregated protrusions. (*A*) Shadowing was with tantalum/platinum/iridium. (*B*) Shadowing was with rhenium/platinum/iridium.

faces, the accuracy of the measurements remains uncertain for several reasons. First, to make the measurements it was necessary to use a relatively high degree of tilt (±10°), which can produce error if not precisely set and translated by the stage tilt drive mechanism. However, an error of 1°, which produces a systematic error of 10% in a calculated height, produces no error in the ratio of particle heights. Second, membranes were rarely flat. Curvature can induce errors in the measurements. Third, we had to select for measurement only particles adjacent to membrane areas that received platinum. We tried to control for these factors by selecting pictures for measurement that had both extracellular and cytoplasmic surfaces exposed on a single vesicle. However, we are unsure if this was a sufficient control. Despite these reservations it is clear that the projection on the cytoplasmic surface is substantial. In future experiments, computer-generated three-dimensional reconstruction should provide an alternative method to obtain particle heights (14).

The complete primary structure of each subunit of the *Torpedo* AChR is known (reviewed in Popot and Changeux, 1984 [33]), and the relative mass distribution of AChR between extracellular and cytoplasmic domains provides an important parameter to constrain topologic models of subunit folding. Most models have assumed that very little receptor mass is present on the cytoplasmic surface. The results reported here, however, are consistent with recent studies (36), based upon the binding of antibodies directed against known receptor sequences that also conclude that a substantial fraction of receptor mass is exposed on the cytoplasmic surface of the postsynaptic membrane.

It is interesting to note that AChRs seem to be able to undergo some lateral redistribution in the membranes that contain 43K protein as indicated by the response to polyornithine-coated glass. This suggests that AChRs are probably not entirely immobilized by interactions with 43K. This also seems to indicate that AChR + 43K protein may move as a

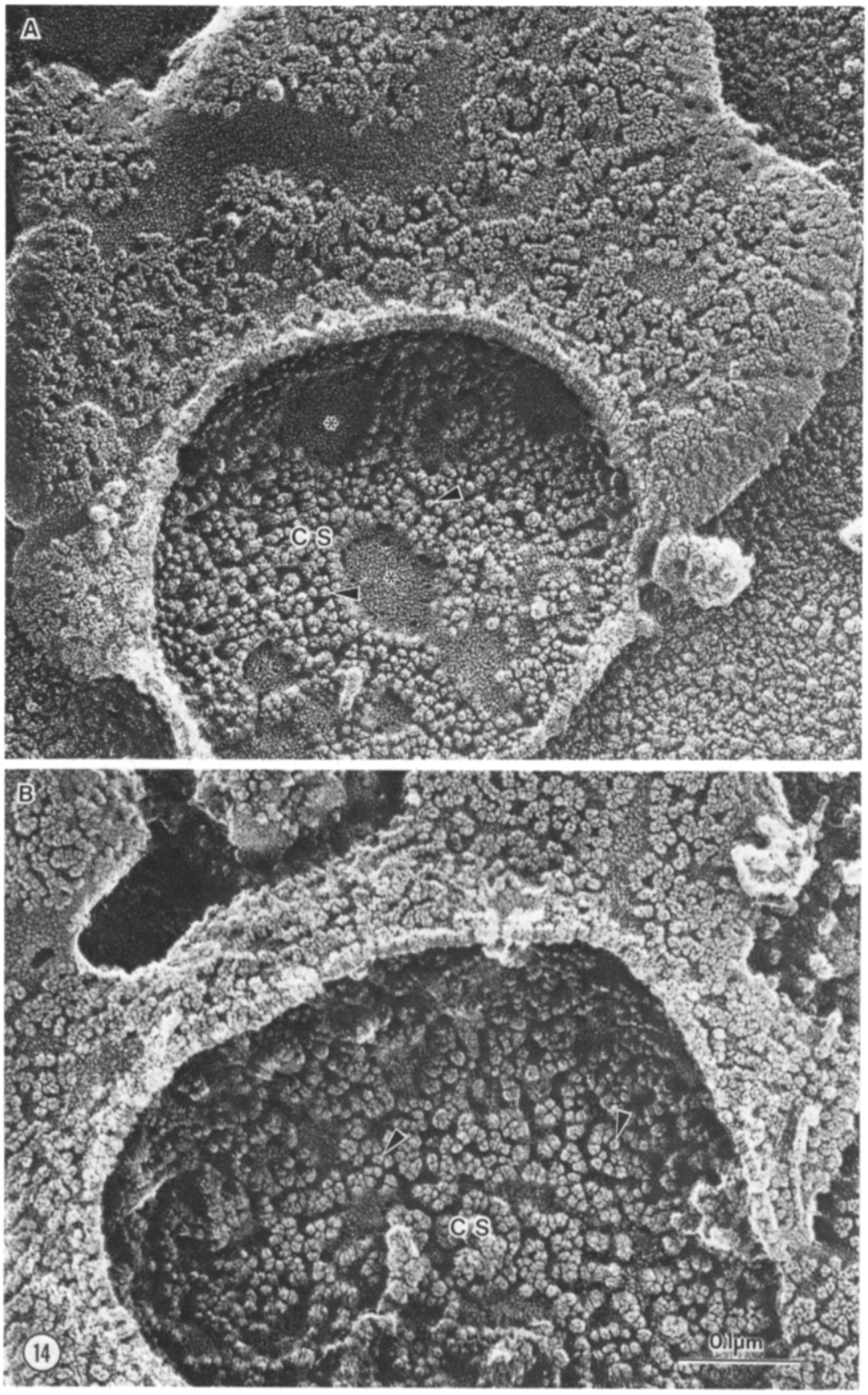

Figure 14. Alkaline-extracted vesicles attached to glass coated with polyornithine (*A*) or Alcian Blue (*B*) and sheared open by sonication to reveal the cytoplasmic membrane surface (*CS*). Adsorption on polyornithine (but not Alcian Blue) results in particle redistribution to form dense aggregates and particle-free surfaces (*asterisks*). Individual protrusions on the cytoplasmic surface (*arrowheads*) are prominent and appear more uniform in size than those seen in unextracted membranes. Shadowing was with rhenium/platinum/iridium.

unit, since rearrangement occurred among all cytoplasmic protrusions, some of which represent the AChR and others that represent either the 43K protein or a complex of the AChR and 43K protein. This redistribution observed in isolated *Torpedo* membranes may be related to the clustering of AChR and 43K protein observed at points of contact between *Xenopus* muscle cells and polyornithine-coated latex beads (32).

The topological distribution of 43K protein as determined by antibody labeling indicates that 43K protein is restricted

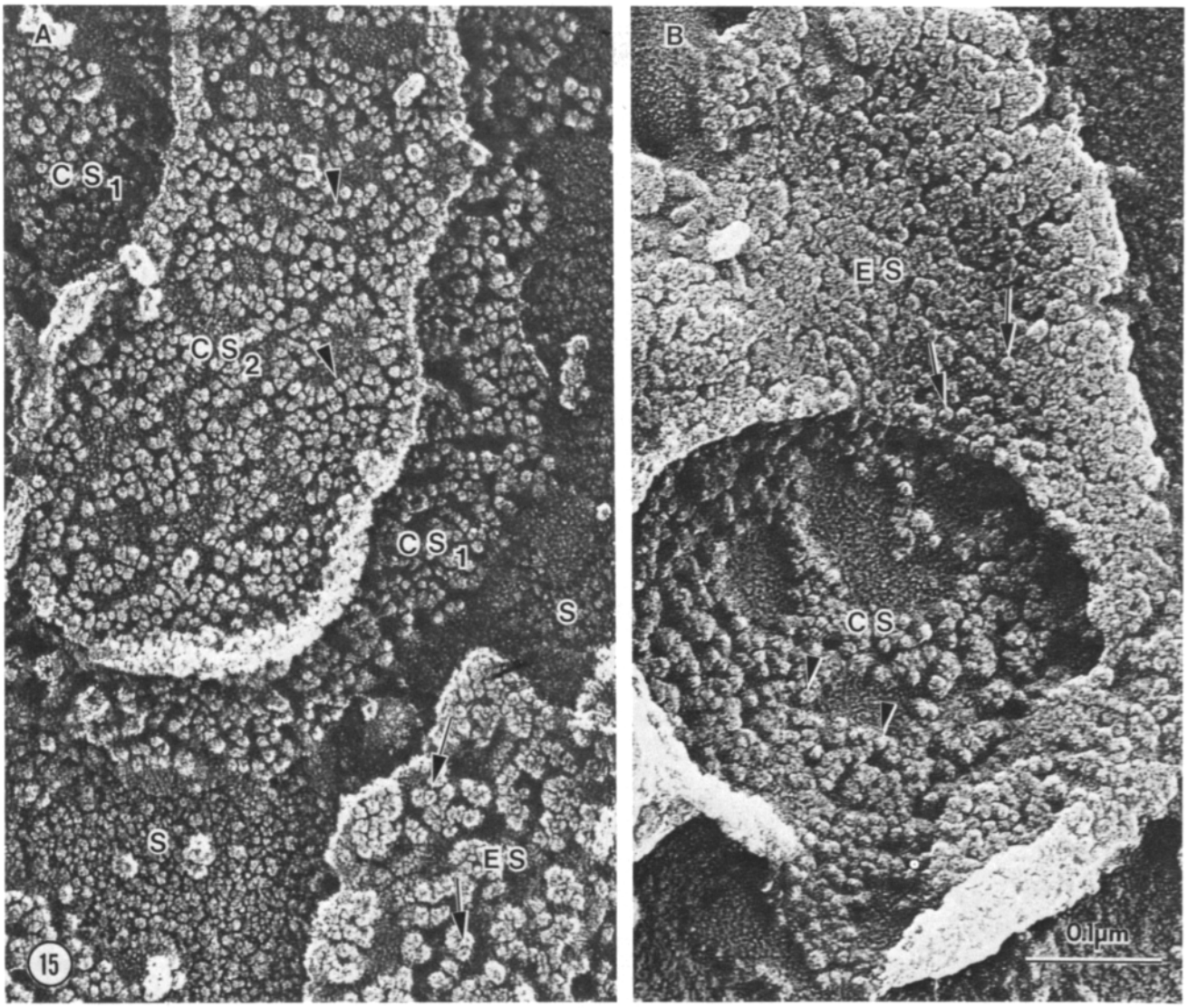

Figure 15. Two different controls for the appearance of cytoplasmic particles in alkaline extracted AChR-rich membranes. (*A*) A control for the effects of the Alcian Blue–coated glass surface on the appearance of cytoplasmic surface particles. A portion of the cytoplasmic surface (CS_2) of a vesicle is resting on top of the cytoplasmic surface (CS_1) of another vesicle. The vesicle is thus separated from the glass surface (*S*) coated with Alcian Blue. Individual cytoplasmic particles (*arrowheads*) appear prominent. For comparison, a portion of the extracellular surface (*ES*) of another vesicle containing AChR particles (*arrows*) is shown. (*B*) A control for decoration effects by the metal coating. A vesicle on Alcian Blue–coated glass was (rotary) preshadowed with 10 Å of carbon before rotary shadowing with platinum/iridium/rhenium. Particles on both the extracellular surface (*ES*) and cytoplasmic surface (*CS*) are prominent. Although the size of particles appears increased and the boundaries are less sharply defined, the relative mass of the cytoplasmic particles (*arrowheads*) compared with extracellular particles (*arrows*) seems the same.

to areas of the membrane containing aggregated AChRs, despite the fact that isolated 43K protein has the capacity to adsorb to many lipid surfaces (35). The stoichiometry of 43K protein to AChR within aggregates is difficult to discern from our results because under the labeling conditions used antibody binding sites were probably far from saturated. Further characterization of antibody affinities and dissociation rate constants would be necessary to evaluate how these parameters affect the labeling density. Because the anti–43K monoclonal appeared in direct association with the cytoplasmic particles, and the surface density of the particles was the same (6,500/μm^2) before and after alkaline extraction, we conclude that 43K protein was associated directly with the particles that remain after alkaline extraction (i.e., the AChR).

The large contribution of the cytoplasmic portion of the AChR to the structure seen on the surface of the membrane complicates the determination of the macromolecular structure of 43K protein. Hopefully, rotary-shadowed preparations of purified 43K protein will allow a more exact determination of its structure.

In this study we have established the contribution of AChR and 43K protein to the observed cytoplasmic projection of the *Torpedo* nicotinic postsynaptic membrane. However, it is

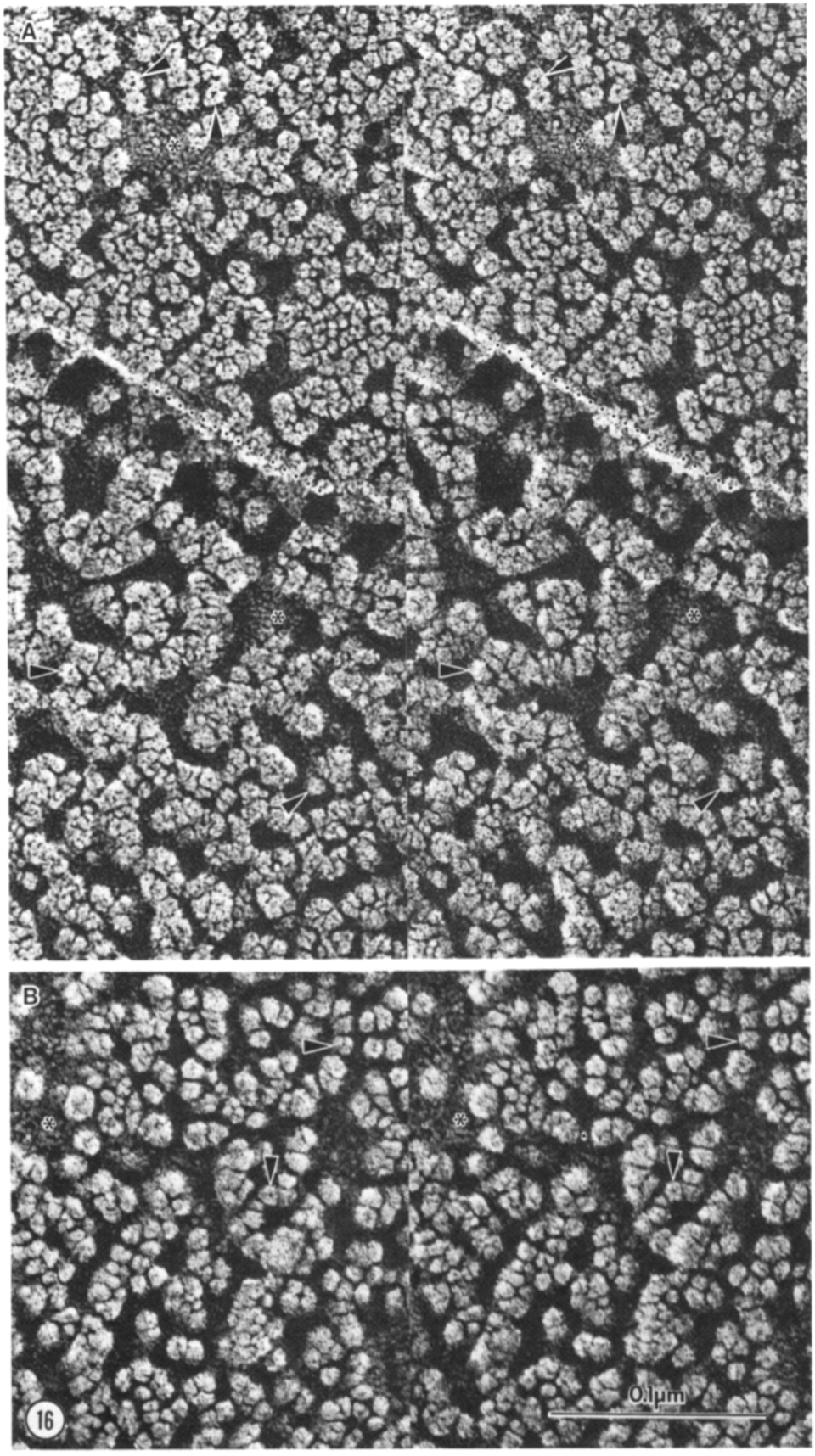

Figure 16. High magnification stereo views of AChR-rich membrane surfaces. (*A*) From a vesicle that was attached to the glass surface with Alcian Blue and then sheared open by sonication. The upper portion of the image (above dots) shows a portion of the extracellular surface. In favorable orientations the particles appear doughnut-shaped (*arrowheads*). The valleys between aggregated particles accumulate platinum only when the space is relatively large (*asterisk*). The lower portion of the image (below dots) shows the cytoplasmic surface. Individual particles (*arrowheads*) are less easily recognized and on the average appear to project further from the membrane surface (*asterisk*) than extracellular particles. (*B*) The cytoplasmic surface of an alkaline extracted vesicle attached to glass using Alcian Blue. Individual cytoplasmic particles (*arrowheads*) are now more easily recognized and appear to project about the same distance from the membrane surface (*asterisk*) as extracellular particles.

Table I. Torpedo Postsynaptic Membrane: Extracellular and Cytoplasmic Projection Heights

Prep. No.	Extracellular protrusion height	Cytoplasmic protrusion height	*n*	hep/hcp
	nm ± SD	*nm ±SD*		*± SD*
ACh Receptor-rich vesicles				
1. (PO)	5.5 ± 0.5	6.6 ± 1.1	(30)	0.83 ± 0.16
2. (PO)	5.1 ± 0.5	6.3 ± 1.4	(30)	0.81 ± 0.20
3. (PO)	4.8 ± 0.4	5.8 ± 1.2	(30)	0.83 ± 0.19
4. (AB)	4.5 ± 0.6	6.3 ± 1.4	(30)	0.68 ± 0.18
Average	5.0	6.3		0.79 ± 0.09
Alkaline-extracted ACh receptor-rich vesicles				
5. (PO)	5.0 ± 0.4	5.0 ± 0.5	(30)	1.00 ± 0.13
6. (PO)	4.9 ± 0.4	4.6 ± 0.4	(30)	1.07 ± 0.13
7. (PO)	5.3 ± 0.6	5.3 ± 0.5	(30)	1.00 ± 0.15
8. (AB)	5.1 ± 0.5	5.5 ± 0.5	(30)	0.93 ± 0.12
Average	5.1	5.1		1.00 ± 0.07

Measurements were made on eight different replicas. For each preparation a single vesicle that had portions of both extracellular surface and cytoplasmic surface was chosen. For instance, protrusions on the vesicle shown in Fig. 5 were measured and are indicated above as Prep. No. 2. Only clearly recognizable particles were chosen for measurement. (PO) indicates vesicles on polyornithine, whereas (AB) indicates those on Alcian Blue. hep, average height of extracellular projection; hcp, average height of cytoplasmic projection.

also recognized that other nonreceptor proteins are present at the cytoplasmic surface of the *Torpedo* membranes but at apparently lower concentrations than 43K protein. Froehner (15) described a protein of M_r 58,000 present in *Torpedo* membranes and at vertebrate endplates, and we have established that this protein as well as another of M_r 80,000 are present at the cytoplasmic surface of *Torpedo* membranes and in association with AChR clusters in primary cultures of chick muscle (Cohen, J. B., C. Carr, and G. D. Fischbach, manuscript in preparation). Hopefully, the techniques described here can be used to determine the relative contribution of 43K protein and these novel proteins to the structures observed on the cytoplasmic surface of postsynaptic *Torpedo* membranes.

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