Nicotinic Postsynaptic Membranes from *Torpedo*: Sidedness, Permeability to Macromolecules, and Topography of Major Polypeptides

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ABSTRACT Experiments were conducted to examine the topographic arrangement of the polypeptides of the acetylcholine receptor (AcChR) and the nonreceptor M₄ 43,000 protein in postsynaptic membranes isolated from *Torpedo* electric organ. When examined by electron microscopy, >85% of vesicles were not permeable to ferritin or lactoperoxidase (LPO). Exposure to saponin was identified as a suitable procedure to permeabilize the vesicles to macromolecules with minimal alteration of vesicle size or ultrastructure. The sidedness of vesicles was examined morphologically and biochemically. Comparison of the distribution of intramembrane particles on freeze-fractured vesicles and the distribution found in situ indicated that >85% of the vesicles were extracellular-side out. Vesicles labeled with α-bungarotoxin (α-BgTx) were reacted with antibodies against α-BgTx or against purified AcChR of *Torpedo*. Bound antibodies were detected by the use of ferritin-conjugated goat anti-rabbit antibody and were located on the outside of >99% of labeled vesicles. Similar results were obtained for normal vesicles or vesicles exposed to saponin. Quantification of the amount of [³H]-α-BgTx bound to vesicles before and after they were made permeable with saponin indicated that <5% of α-BgTx binding sites were cryptic in normal vesicles. It was concluded that >95% of postsynaptic membranes were oriented extracellular-side out.

LPO-catalyzed radioiodinations were performed on normal and saponin-treated vesicles and on vesicles from which the M₄ 43,000 protein had been removed by alkaline extraction. In normal vesicles, polypeptides of the AcChR were iodinated while the M₄ 43,000 protein was not. In vesicles made permeable with saponin, the pattern of labeling of AcChR polypeptides was unchanged, but the M₄ 43,000 protein was heavily iodinated. The relative iodination of AcChR polypeptides was unchanged in membranes equilibrated with agonist or with α-BgTx or after alkaline-extraction. It was concluded that the M₄ 43,000 protein is present on the intracellular surface of the postsynaptic membrane and that AcChR polypeptides are exposed on the extracellular surface.

Analysis of the mechanism of permeability control by nicotinic cholinergic receptors is facilitated by the isolation of the plasma membrane of the innervated *Torpedo* electrocyte surface, which is highly enriched in nicotinic receptors (for a review, see reference 1). The nicotinic receptor (AcChR), which constitutes ~50% of the protein in the purified postsynaptic membrane, is composed of polypeptides of apparent molecular weights of 40,000 (α), 50,000 (β), 60,000 (γ), and 65,000 (δ) (2-5). When analyzed by SDS PAGE, the isolated membranes contain in addition to those polypeptides only one predominant peptide or M₄ (relative molecular mass) 43,000 (6-10). The membranes appear in the electron microscope as a heterogeneous population of vesicles and membrane fragments with dimensions ranging from <0.1 μm to 1 μm (10-12). Some of the vesicles are sealed to ²²Na⁺, and analysis of the agonist-induced efflux of ²²Na⁺ indicates that the AcChRs in the vesicles probably
transport ions as efficiently as receptors in intact cells (13, 14). The M₄3,000 protein can be removed from the vesicles by alkaline extraction (9) without altering the known binding properties of cholinergic ligands (agonists, competitive antagonists, and local anesthetics) or of the permeability response itself (9, 15-17). Although the M₄3,000 protein is not necessary for permeability control, its removal from the membranes is associated with an increase in rotational mobility of the AChR (18, 19) and of its sensitivity to toxins (20, 21) and thermal denaturation (22). Antibodies directed against the M₄3,000 protein bind to antigenic determinants concentrated at the innervated surface of the Torpedo electrocyte and also at the vertebrate neuromuscular junction (23). These results suggest that the M₄3,000 protein has a structural function in nicotinic postsynaptic membranes.

Information about vectorial orientation of the peptides of the Torpedo postsynaptic membrane could be obtained by the use of the lactoperoxidase (LPO)-catalyzed iodination of membrane proteins (24, 25). Prerequisite to these studies are knowledge that the vesicles possess a common orientation and are impermeable to LPO and the availability of methods for permeabilizing the vesicles without affecting their orientation. One way in which the orientation of the vesicles can be defined is by the location of the acetylcholine (a-bungarotoxin) binding sites. Strader and Rafferty (12) used immunocytochemical methods to localize bound a-bungarotoxin (a-BgTx) and concluded that >95% of the sites were on the outside of vesicles. Huang (26), using a different membrane isolation procedure, concluded that only 50% of the vesicles were sealed with their binding sites on the outside.

We report here the use of electron microscopic techniques to characterize the sidedness and sealing of Torpedo postsynaptic membranes isolated according to the procedure of Sobel et al. (6). More than 90% of the vesicles were found to be oriented with the extracellular-side out and sealed to LPO. To permit entry of macromolecules into the vesicles, various methods were tested including osmotic shock, sonication, and treatment with detergents. Exposure to low concentrations of saponin, which is known to complex with cholesterol (27), was found to increase vesicle permeability to proteins with minimal disruption of ultrastructure. When LPO-catalyzed iodination was performed on native and on saponin-treated vesicles, peptides of the AChR were similarly iodinated in both cases, but the M₄3,000 protein was iodinated only after permeabilization. Preliminary reports of these studies have appeared (28, 29).

MATERIALS AND METHODS

Membrane Preparation and Characterization

Nicotinic postsynaptic membranes were isolated from freshly dissected Torpedo electric tissue by the method of Sobel et al. (6) with minor modifications (10). The final fractionation step involved centrifugation on a continuous sucrose gradient formed by two cycles of freezing and thawing of a 35% sucrose-0.02% NaN₃ solution. Fractions from that gradient were collected and assayed for the binding of [³H] or [¹²⁵I]a-BgTx by the method of Weber and Changeux (30), as modified by Neubig and Cohen (31), and for protein by the method of Lowry et al. (32). For nine fish (eight Torpedo californica and one T. nobiliana) the specific activity of the most enriched fraction (38% [wt/wt] sucrose) was 2.1 ± 0.5 nmol a-toxin/g protein. Membrane suspensions were stored at 4°C in 38% sucrose-0.02% NaN₃. Non-AChR peptides were removed from the isolated Torpedo membranes by alkaline treatment at 4°C according to the method of Neubig et al. (9). The peptide composition of the isolated membranes was determined by electrophoresis in polyacrylamide slab gels containing SDS according to the method of Lasemlini (33). The separating gel contained 8% acrylamide (Sigma Chemical Co., St. Louis, Mo.), Gels were stained with Coomassie Brilliant Blue R.

Permeability of Isolated Torpedo Vesicles to Macromolecules

The permeability of membranes to LPO (Sigma Chemical Co.) was determined by thin-section electron microscopy as follows. Membrane suspensions were diluted to 1 mg protein/ml in Torpedo physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 5 mM Na₂HPO₄, pH 7, 0.02% NaN₃). LPO was dissolved in TPS at 10 mg protein/ml. Membranes and LPO (0.1 ml each, were mixed in a polyethylene capsule (BEEM Products from Ernest F. Fullam, Inc., Schenectady, N. Y.) and incubated at 4°C for 30 min before 0.1 ml of fixative solution (4% glutaraldehyde in cacodylate, pH 7.4) was added. Membranes were incubated for an additional 20 min and then centrifuged for 20 min at 20,000 g. Pellets were washed overnight in 20 mM Tris-Cl, pH 7.0-7.2. After this, pellets were incubated for 30 min in 0.5 M NaCl/ml of 3.3'-diaminobenzidine (DAB; Sigma Chemical Co.) and then for 1 h in 0.5 mg/ml DAB containing 0.001% H₂O₂ (34). After incubation with DAB, samples were washed, then immersed in 1% OsO₄ for 60 min at 4°C, stained in block with 1% aqueous uranyl acetate for 60 min, dehydrated in ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a Siemens 101 electron microscope operating at 80 kV.

Different physical perturbations were examined that might disrupt the integrity of the bilayer and thereby permit entry of macromolecules into Torpedo vesicles. Unless otherwise noted, all procedures were carried out at 4°C. Membranes were treated with detergent as follows. Membranes (9 vol; 1 mg protein/ml in TPS) were mixed with 1 vol of detergent in water and then incubated for 30 min. The membranes were then centrifuged for 30 min at 194,000 g, resuspended in TPS at 1 mg protein/ml, and rechromatographed. The pellet was then resuspended in TPS. Membrane permeability and ultrastructure were assessed as described above. Detergents examined at final concentrations of 0.01-0.1% (wt/vol) included digitonin (J. T. Baker, Chemical Co., Phillipsburg, N. J.), saponin (Sigma Chemical Co.), and Triton X-100. For freeze/thaw experiments, membrane suspensions in 38% sucrose were mixed with an equal volume of 10 mM Tris-Cl solution containing LPO (10 mg/ml). That mixture was subjected to two cycles of freezing at ~20°C and thawing on ice. For osmotic shock, membranes in 38% sucrose were diluted rapidly in 10 vol of 5 mM Tris-Cl (pH 7.0) containing LPO (5 mg/ml). For sonication, 0.2 ml of a membrane suspension in 38% sucrose was mixed with an equal volume of LPO (10 mg/ml) in 5 mM Tris-Cl (pH 7.0) and sonicated for 15 s with the microtip of a Branson sonifier operating at 80 W (Branson Sonic Power Co., Danbury, Conn.).

Freeze-fracture and Freeze-etch

Electron Microscopy

Small pieces of tissue were removed from the electric organ, minced, and fixed for 30-60 min in fixative (4% glutaraldehyde in 50 mM cacodylate, pH 7.0). After fixation, the material was immersed in 5%, 10%, and 20% glycerol for 30 min each, and then in 30% glycerol for freezing. Small pieces of tissue were transferred to 3-mm paper disks and frozen in liquid freon cooled by liquid nitrogen (~150°C). Isolated membranes for freeze-fracture or freeze-etch were diluted in 5 mM sodium phosphate, pH 7 or 5 mM Tris, pH 7, and were centrifuged for 20 min at 20,000 g. Membranes for freeze-fracture were resuspended in 20-25% sucrose as before. Small aliquots of the pellet were transferred with a small boro pipette to paper disks and were frozen as described above. Frozen samples were fractured in a Balzers freeze-etch apparatus (BA 360 M, Balzers, Hudson, N. H.) at ~115°C to ~120°C and were immediately shadowed with platinum and carbon. Sizes and densities of intramembrane particles in freeze-fractured membranes were measured on photographic prints at a final magnification of ×90,000-180,000. Diameters on freeze-fractured and freeze-etch material were measured in a direction perpendicular to the direction of platinum shadow.

Labeling of Membranes with Antisera

Thin-section electron microscopy was used to localize the binding to the isolated Torpedo postsynaptic membranes of anti-a-sera directed against a-BgTx and against detergent-solubilized, purified AChR. The presence of bound antibodies was revealed by the use of ferritin-conjugated, goat anti-rabbit antibodies. Preparation and characterization of the anti-sera have been presented elsewhere (35). a-Bungarotoxin binding was localized as follows. Membranes (1-2 nmol a-BgTx binding sites/mg protein) were diluted to approximately 1 mg protein/ml in TPS and were incubated at 23°C for 3 h with a fivefold excess of a-BgTx (Miami Serpentarium Labs, Miami, Fla.). Membranes were centrifuged and resuspended twice in TPS (20 min at 20,000 g) to remove unbound a-BgTx. For each sample a 0.1-ml aliquot was transferred to a 1.5-ml centrifuge tube with

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Alteration of Vesicle Permeability

Attempts were made to alter the permeability of vesicles to LPO or ferritin using osmotic shock, sonication, and cycles of freezing and thawing. Most conditions tested produced no detectable change in permeability. The results of one experiment are summarized in Table II. Although the permeability of the vesicles used in this experiment (15%) was at the high end of the range for all preparations, it was substantially decreased by only one treatment: sonication of membranes in the presence of LPO. Even this treatment, which caused a decrease in the average diameter of the vesicle population from ~0.32 μm to ~0.15 μm, left >25% of the vesicles impermeable.

The effects of detergents on membrane ultrastructure and vesicle sealing were examined following a standard experimental protocol (see Materials and Methods). Exposure of vesicles to 0.01% Triton X-100 neither perturbed membrane ultrastructure nor increased the fraction of vesicles permeable to LPO. Since that concentration was known to have a profound effect on functional properties of nicotinic receptors (37, 38), attention was focused on other detergents. Saponin and digitonin were found to cause a concentration-dependent increase in the permeability of the vesicles to LPO and, of the two, saponin produced that effect without grossly altering the vesicle morphology. In a typical experiment, 0.01% and 0.03% digitonin permeabilized, respectively, ~50% and >95% of the vesicles, but 0.03% digitonin resulted in membranes which appeared in thin-section more as fragments than closed vesicles. Exposure to saponin produced vesicles which retained their vesicular form (Fig. 2), although breaks were apparent in the trilaminar structure. While 0.01% saponin permeabilized 75% of the vesicles to LPO, 0.03% and 0.1% permeabilized 95% and >98%, respectively (Fig. 4). Following exposure to saponin, the vesicles were permeable to ferritin (M, 400,000) as well (see below). Saponin treatment did not otherwise affect the structural or functional properties of the AcChR-rich membranes as determined by five criteria. Saponin-treated membranes retained 93 ± 9% of the protein and 100 ± 19% of the α-BgTx binding capacity of control membranes. The polypeptide composition of saponin-treated membranes was the same, within the resolution of the gel techniques, as that of control membranes. In addition, saponin-treated membranes retained the rosettes characteristic of negatively stained control membranes (see references 11, 39) and the asymmetric distribution of intramembrane particles seen in freeze-fracture (see below). Finally, normal and saponin-treated membranes undergo the same degree of agonist-induced conformational transition as monitored by the kinetics of binding of [125I]α-BgTx (35).

Sidedness of Isolated Torpedo Vesicles

The sidedness of the vesicles was examined in four ways. We first took advantage of the fact that the intramembrane particles (IMPs) seen in freeze-fractured membranes of Torpedo electrocytes are asymmetrically distributed between the two leaflets of the membrane (11, 40–42). On both the innervated (ventral) and noninnervated sides of the cell, the E-face (43) contains a high density of large IMPs, while the F-face contains a low density of large IMPs (41 and Table III). Large IMPs also were asymmetrically distributed on the membranes of isolated vesicles (Fig. 5). Because both the innervated and the noninnervated sides of electrocytes in situ had large IMPs of nearly the same size and density (Table III), an isolated vesicle could not be identified as a fragment of either innervated or noninnervated membrane by these criteria. Nevertheless, the

LPO-catalyzed Iodination of Torpedo Membranes

The LPO-catalyzed iodination of membranes was performed according to a method described by Morrison (24). When membranes were to be iodinated without modification of their permeability properties, Torpedo vesicles which had been stored in 38% sucrose-0.02% NaN₃ were transferred without pelleting to iodination buffer (150 mM NaCl, 5 mM KCl, 20 mM NaPO₄, pH 7.2) by passage over a 1.0 × 15-cm column of Sephadex G-50 equilibrated with iodination buffer. In preliminary experiments it was established that >95% of the postsynaptic membranes (α-BgTx binding sites) were recovered in the void volume. Membranes which had been treated with saponin and/or alkaline pH were then resuspended in iodination buffer at the same protein concentration as untreated membranes. For a typical iodination, 450 μl of membranes (1.0–1.3 mg/ml) was mixed with 5 μl of LPO (Sigma Chemical Co.; prepared at 1 mg/ml in iodination buffer). Control membranes were incubated under the same conditions as other samples, but in the absence of LPO. 25 μl of Na[1211] (50 ACi, New England Nuclear, Boston, Mass.) was added to each sample immediately before the reaction was started. H₂O₂, prepared by dilution of a 30% stock to 0.1 μM in iodination buffer, was added in 10-μl aliquots to each sample every 5 min during the reaction. After an incubation time of 65 min, each reaction was stopped by the addition of 100 μl of 5 mM Na₂SO₄, and each sample was then passed over a 1.0 × 15-cm column of Sephadex G-50 that was eluted with iodination buffer containing 0.1 mM Na₂SO₄. The material eluting in the void volumes of the columns was centrifuged for 30 min at 150,000 g. Each pellet was resuspended in iodination buffer at a protein concentration of ~1 mg/ml and then centrifuged again under the same conditions. Each pellet was resuspended in water at 2 mg protein/ml and kept at 4°C until electrophoresis.

RESULTS

Permeability of Vesicles to Macromolecules

When examined by thin-section electron microscopy, the isolated AcChR-rich membranes appear vesicular in shape with an unbroken, trilaminar ultrastructure (Fig. 1). The permeability of vesicles to LPO was determined by electron microscopic localization of the enzymatic reaction product after LPO had been covalently attached to the membranes by fixation. Vesicles with reaction product localized only on the exterior surface of the bilayer (Fig. 3) were concluded to have been impermeable to LPO, while vesicles with reaction product on both sides (Fig. 4) were permeable (36). In a typical experiment, permeability to LPO could be determined for ~95% (n = 83) of the vesicles that had been cut in cross-section. In experiments with membranes prepared from four fish, the proportion of vesicles impermeable to LPO varied from 80% to >95% (Table I). For each suspension, no significant change in sealing to LPO was observed when examined at times varying from 5 d to 3 wk after the initial preparation. In two cases, permeability to LPO was also measured after alkaline-extraction (pH 11, 1 h at 4°C) to remove the M, 43,000 protein. In one experiment, ~80% of the vesicles were impermeable before and after alkaline extraction, while in the second case 90% were impermeable before, but only 30% after. The cause of this variability is unknown, but it demonstrates that alkaline-extraction can alter dramatically the integrity of the bilayer.
**TABLE I**

**Permeability to LPO of Native and Alkaline-extracted Torpedo Vesicles**

| Preparation sp act | Condition (days after initial isolation) | % Vesicles impermeable to LPO (n = number scored) |
|--------------------|------------------------------------------|--------------------------------------------------|
| μmol a-BgTx/g protein |                                         |                                                  |
| A: 2.7             | Native (7 d)                             | 80 (n = 75)                                      |
|                    | Native (23 d)                            | 79 (n = 109)                                    |
|                    | Alkaline extracted (25 d)                | 75 (n = 73)                                      |
| B: 1.9             | Native (3 d)                             | 90 (n = 68)                                      |
|                    | Native (21 d)                            | 95 (n = 63)                                      |
|                    | Native (5 d)                             | 95 (n = 54)                                      |
|                    | Native (14 d)                            | 92 (n = 79)                                      |
|                    | Alkaline extracted (14 d)                | 30 (n = 47)                                      |

**TABLE II**

**Effect of Osmotic Shock, Sonication, and Freeze/Thaw on Permeability of Torpedo Vesicles to LPO**

| Treatment                        | % of vesicles impermeable to LPO |
|----------------------------------|----------------------------------|
| Control                          | 85 (n = 47)                      |
| Freeze/thaw before LPO           | 86 (n = 44)                      |
| Freeze/thaw in LPO               | 79 (n = 51)                      |
| Sonication in LPO                | 26 (n = 116)                     |
| Osmotic shock before LPO         | 68 (n = 38)                      |
| Osmotic shock in LPO             | 67 (n = 73)                      |

* Results of a single experiment in which all samples were prepared from the same starting preparation of vesicles (Preparation A of Table I).

**TABLE III**

**Intramembrane Particle (IMP) Sizes and Densities from Freeze-fracture of Torpedo Electric Tissue and Isolated Vesicles**

| Location                  | Face Diameter | Density Area counted |
|---------------------------|---------------|----------------------|
| Ventrail (innervated surface) | P 10.8 ± 1.9  | 4,010 0.37          |
| Dorsal (noninnervated surface) | E 8.9 ± 1.3  | 320 0.32             |
| Isolated vesicles         | P 12.1 ± 1.7  | 3,650 0.19           |
| Saponin-treated vesicles  | P 11.3 ± 2.6  | 2,450 0.25           |
| E 10.7 ± 2.1              | 850 0.15      |
| ND 2,350                  | 1,200         |

ND, not determined.

**FIGURES 1-4**

Figs. 1-4 illustrate the impermeability of most native AChR-rich vesicles to LPO and the increase in permeability caused by saponin. Fig. 1: native vesicles with no LPO. Fig. 2: vesicles treated with 0.03% saponin for 30 min, then prepared for electron microscopy with no LPO; arrows indicate breaks in trilaminar membrane. Fig. 3: native vesicles mixed with LPO; then fixed with glutaraldehyde before pelleting and subsequent reaction with DAB (see Materials and Methods). LPO-catalyzed reaction product is uniformly distributed around outside of trilaminar membranes (arrows); in this field, only the vesicles marked with asterisks could be scored and would be considered not permeable to LPO. Fig. 4: vesicles treated with 0.03% saponin, then prepared with LPO. Reaction product is found on both sides of trilaminar membranes (arrows). Only the vesicles marked with asterisks could be scored and they would be considered permeable to LPO. Bars, 100 nm. Fig. 1: ×95,000. Fig. 2: ×110,000. Fig. 3: ×105,000. Fig. 4: ×105,000.

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**43,000 Protein in Torpedo Membranes**

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**Lactoperoxidase-catalyzed Iodination of Vesicle Peptides**

We used protein iodination catalyzed by LPO to determine the topography of the major membrane proteins, namely the subunits of the AcChR and the M, 43,000 protein. The vesicles used in these studies were shown above to be 95% extracellular-side out. Incorporation of $^{125}$I into the polypeptides of normal membrane vesicles impermeable to LPO was compared to that of vesicles permeabilized by 0.1% saponin (Fig. 11). In control experiments we established that $^{125}$I incorporation was dependent upon the presence of both H$_2$O$_2$ and LPO, indicating that the iodination was enzymatic. In normal membranes, heavy labeling was found on the α-chain of the AcChR (M, 41,000) and some incorporation also was detected on the β (M, 49,000) and δ (M, 65,000) subunits. While the receptor peptides were iodinated, there was no incorporation of $^{125}$I into the M, 43,000 protein. In addition to the iodination of peptides of postsynaptic membranes, there was incorporation of $^{125}$I into peptides of ~95,000 daltons, which are associated with contaminating membrane fractions (9, 10), and incorporation into a minor peptide of M, 47,000 (gel slice 42). Recent evidence suggests that this latter component is a degradation product of one or more of the large receptors subunits (23). When the vesicles were made permeable to LPO by the use of saponin, the relative incorporation of $^{125}$I into the receptor chains was

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**FIGURES 6-9** Labeling of AcChR-rich vesicles with antisera against α-BgTx or against purified Torpedo AcChR. Ferritin molecules are indicated by arrows. Fig. 6: native vesicles were incubated with excess α-BgTx and washed, then incubated with rabbit antiserum against α-BgTx, followed by ferritin-conjugated goat-anti-rabbit IgG antibodies. Fig. 7: native vesicles incubated with preimmune serum, followed by ferritin-conjugated secondary antibodies as for Fig. 6. Fig. 8: vesicles pretreated with 0.1% saponin for 30 min were labeled with antiserum against purified Torpedo AcChR, followed by ferritin-conjugated secondary antibodies. As described in Results, for a membrane suspension binding 1.9 µmol α-BgTx/g protein, ~80% of vesicles with a diameter >0.2 µm were labeled. Although saponin-treated vesicles are permeable to macromolecules (see text), labeled vesicles were labeled only on the outside. Fig. 9: saponin-treated vesicles mixed with ferritin-conjugated secondary antibodies were fixed without washing. Ferritin molecules were found on insides of vesicles, indicating that reagents for labeling had access to insides of vesicles. Bars, 100 nm. Fig. 6: ×90,000. Fig. 7: ×100,000. Fig. 8: ×90,000. Fig. 9: ×80,000.
The experiments reported here provide a characterization of the sidedness and sealing of AcChR-enriched vesicles isolated from *Torpedo californica* electric organ following homogenization in hypotonic media in the absence of chelators of divalent cations. More than 95% of the vesicles containing AcChRs were found to be oriented with the extracellular-side out. This conclusion is based on the binding of \[^{125}\text{I}]\alpha\text{-BgTx}\) and on electron microscopy analyses after labeling of AcChRs in these membranes with antibodies against \(\alpha\text{-BgTx}\) or against AcChR itself. The conclusion is consistent with results obtained from an analysis of intramembrane particle distribution following freeze-fracture of the isolated vesicles which indicated that >85% of all the isolated vesicles were oriented extracellular-side out.

Strader et al. (12) reported that, following homogenization of *Torpedo* electric tissue in isotonic buffer containing \(\text{Ca}^{2+}\) chelators, 95% of the isolated AcChR-enriched vesicles were also oriented with the extracellular (\(\alpha\text{-BgTx}\) binding site) side out. Since similar results are obtained independent of the tonicity of homogenization buffers or of the presence of diva-

unmodified. In saponin-treated membranes, however, there was heavy iodination of the \(M_r\) 43,000 protein.

In five experiments with two separate membrane preparations in which >95% of the native vesicles were LPO impermeable, iodination of the \(M_r\) 43,000 protein occurred only after saponin-treatment of the membranes. In additional experiments, we examined the LPO-catalyzed iodination of membrane proteins under conditions in which the membranes were equilibrated with cholinergic ligands and also after alteration of membrane structures by removal of the \(M_r\) 43,000 protein (alkaline extraction). In these experiments, we observed that the total amount of \(^{125}\text{I}\) incorporated into polypeptides was not the same for different samples following identical reaction conditions. Although the cause of the variation was not determined, it did not depend upon the particular membrane treatment. Samples with different amounts of \(^{125}\text{I}\) incorporation were compared by plotting the cpm in each gel slice as the percent of the total cpm in the gel above a background level. When results were analyzed in this manner, the patterns of incorporation into duplicate reaction mixtures were the same. The pattern of incorporation of \(^{125}\text{I}\) into subunits of the AcChR was not altered following removal of the \(M_r\) 43,000 protein by alkaline extraction (Fig. 12), and the same relative iodination of the polypeptides was observed for saponin-treated membranes that had subsequently been extracted at pH 11 (data not shown). The pattern of \(^{125}\text{I}\) labeling of normal (LPO impermeable) membranes was not changed by preincubation with excess \(\alpha\text{-BgTx}\). Finally, the \([^{125}\text{I}]\)-labeling pattern was determined in the presence of 0.1 mM carbamylcholine, a concentration sufficient to occupy all AcCh binding sites and to stabilize the desensitized conformation of the receptors. The presence of carbamylcholine did not alter the pattern of labeling of either normal or saponin-treated, alkaline-extracted membranes.

**DISCUSSION**

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The experiments reported here provide a characterization of the sidedness and sealing of AcChR-enriched vesicles isolated from *Torpedo californica* electric organ following homogenization in hypotonic media in the absence of chelators of divalent cations. More than 95% of the vesicles containing AcChRs were found to be oriented with the extracellular-side out. This conclusion is based on the binding of \[^{125}\text{I}]\alpha\text{-BgTx}\) and on electron microscopy analyses after labeling of AcChRs in these membranes with antibodies against \(\alpha\text{-BgTx}\) or against AcChR itself. The conclusion is consistent with results obtained from an analysis of intramembrane particle distribution following freeze-fracture of the isolated vesicles which indicated that >85% of all the isolated vesicles were oriented extracellular-side out.

Strader et al. (12) reported that, following homogenization of *Torpedo* electric tissue in isotonic buffer containing \(\text{Ca}^{2+}\) chelators, 95% of the isolated AcChR-enriched vesicles were also oriented with the extracellular (\(\alpha\text{-BgTx}\) binding site) side out. Since similar results are obtained independent of the tonicity of homogenization buffers or of the presence of diva-
of the Torpedo vesicles to LPO as judged by the presence of the diamino-benzidine reaction product on the inner surface. Stress conditions that resulted in significant permeabilization equilibration was observed. We were unable to identify osmotic and, for a molecule such as inulin (Mr = 5,000), only incomplete shock to facilitate the entry of $^{22}$Na$^+$ and $[^3H]$sucrose into oxidase but also of immunoglobins and ferritin-conjugated to permit entry of macromolecules to the inside of the vesicles. Stated for extended periods at 4°C. We examined different conditions that apparently permitted access of trypsin (Mr, 24,000) to the inside of vesicles, though no ultrastructural characterization of the vesicles was reported.

After exposure to 0.03–0.1% saponin, and then pelleting and resuspension in buffer containing no free detergent, >95% of the Torpedo vesicles remained permeable to LPO. Treatment with saponin, which interacts specifically with cholesterol (27), did not alter the distribution of IMPs in freeze fracture or the polypeptide composition of the membranes. Neither the amount of $\alpha$-BgTx binding nor the agonist-induced conformational changes which can be measured from the kinetics of $[^125I]$ $\alpha$-BgTx binding (35) appeared perturbed. The only evidence that saponin caused a structural change in the membrane other than a disruption of the permeability barrier was the appearance of discrete breaks in the trilaminar ultrastructure of the membrane and the fact that saponin treatment apparently interfered with the binding of antibodies against $\alpha$-BgTx to $\alpha$-BgTx on the membranes. The binding of anti-AcChR antibodies was not similarly inhibited.

When normal and saponin-treated membranes were used as substrates for LPO-catalyzed protein iodination, the non-AcChR, Mr 43,000 protein was significantly iodinated only in membranes that had been made permeable to LPO with saponin. A reasonable interpretation of these results is that the Mr 43,000 protein has tyrosine residues exposed only on the intracellular surface of the postsynaptic membrane and has no such residues on the extracellular surface. The fact that this polypeptide can be extracted from the membrane by alkali (9) suggests that it is not an integral membrane protein and does not span the membrane. Recent immunofluorescence experiments using an antiserum directed against the Mr 43,000 protein indicate that this component is restricted to the innervated membrane of the Torpedo electrocyte (23). Therefore, the Mr 43,000 protein is probably a peripheral membrane protein located on the intracellular surface of the postsynaptic membrane.

This conclusion concerning the location of the Mr 43,000 protein is the same as that recently reached by Wennogle and Changeux (20). On the basis of proteolytic treatment of AcChR-rich membranes from Torpedo marmorata, they concluded that the Mr 43,000 protein is exposed only on the intracellular surface of the membrane. Such a location is interesting in light of reports that extraction of membranes at pH 11, which removes the Mr 43,000 protein, increases the rotational mobility (19, 29) and the thermal sensitivity (22) of the AcChR. An immunologically related component of rat muscle endplates also appears to be intracellular (23, 47). In immunofluorescence experiments, antibodies against the Torpedo Mr 43,000 protein do not label neuromuscular junctions when incubated with intact muscles, while anti-AcChR antibodies do. In cryostat sections of muscle fibers, in which the intracellular face of the postsynaptic-membrane is accessible to antibodies, the endplates are labeled with anti-Mr 43,000 antiseras.

The iodination results suggest that the $\alpha$, $\beta$, and $\delta$ subunits of the AcChR are exposed on the extracellular surface of the membrane, since they were iodinated even in membranes impermeable to LPO. This conclusion is consistent with results obtained by other experimental methodologies. Each of these

**Figure 12** LPO-catalyzed incorporation of $^{125}$I into polypeptides of native vesicles and vesicles from which the Mr, 43,000 protein had been removed by alkaline extraction. Data were collected as for Fig. 11. Because the samples had different total amounts of $^{125}$I incorporated, results for the samples were normalized. For each slice, cpm above the background were expressed as percent of the total for all slices in the sample; the background for each sample was defined as cpm present in a region of the gel containing no stained protein, usually in the region between Mr, 65,000 and Mr, 95,000. Apparent molecular weights of major bands, expressed in thousands of daltons, are indicated at top. T.D., tracking dye.

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polypeptides contains carbohydrate residues (5, 20) which are presumed to lie on the extracellular surface of the membrane (48). Antibodies binding to these subunits are absorbed by the sealed Torpedo vesicles (35), thus indicating the existence of antigenic sites on the outer surface. The LPO-catalyzed incorporation of [125I] into the peptides of the AcChR was not modified by saponin-treatment alone or by alkaline extraction in conjunction with saponin permeabilization. These treatments do expose antigenic determinants of the AcChR that are not accessible in intact vesicles (35). Since it is estimated that 85% of the mass of the AcChR is outside the bilayer (39), the LPO-catalyzed iodination of the inner surface of the vesicles will be more readily quantified when the reaction is carried out after removal of LPO from the external solutions. Experiments of this sort are underway to iodinate regions of the AcChR exposed after removal of the M, 43,000 protein.

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