DEMONSTRATION OF THE TRANSMEMBRANE NATURE OF THE ACETYLCHOLINE RECEPTOR BY LABELING WITH ANTI-RECEPTOR ANTIBODIES

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ABSTRACT

Antibodies raised in rabbits to Triton-solubilized, purified acetylcholine receptor from *Torpedo californica* were used to immunospecifically label intact *T. californica* electroplaque membrane vesicles attached to cover slips and oriented with the extracellular face of the synaptic membrane facing outward. Hemocyanin conjugated to Protein A was then used as a marker, making the antibody binding visible at the electron microscope level. Parallel labeling experiments were performed on vesicles attached to cover slips and sheared by sonication, leaving their cytoplasmic faces fully exposed to the labeling solution. While differences in antibody populations among different rabbits were observed, antigenic determinants of the receptor were present on both faces of the membrane, with those on the extracellular side more numerous than those on the cytoplasmic side, demonstrating the transmembrane nature of the receptor molecule.

KEY WORDS receptor · transmembrane · acetylcholine · antibodies · electron microscopy

Membrane fragments containing up to 50% of their total protein as acetylcholine receptor (AcChR)[1] can be isolated from the electric organs of *Torpedo californica* (6, 8). Such membrane fragments have most, if not all, of the molecular properties of the postsynaptic membrane of the electroplaques; they bind α-neurotoxins (6, 8) and cholinergic ligands (27, 41) at structurally related sites and possess distinct binding sites for local anesthetics (7, 34, 41) and the alkaloid histrionicotoxin (10, 11). Cholinergic agonists binding to the AcChR cause the flux of inorganic cations through the membrane (4, 13, 17, 23), suggesting that the AcChR is all or part of a transmembrane complex. The question of the disposition of the AcChR molecule in the membrane, however, remains a controversial issue.

When examined at the electron microscope level, AcChR-enriched membrane fragments are observed to be mostly in the form of vesicles, with densely packed cylindrical rosettes 60–80 Å in diameter (3, 24, 28) that become more prevalent

[1] *Abbreviations used in this paper*: AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; EAMG, experimental autoimmune myasthenia gravis; Hcy/A, hemocyanin-Protein A conjugate; Ig, immunoglobulin; MFTS, Triton-solubilized membrane fragment proteins; MFTS-R, Triton-solubilized membrane fragment proteins depleted in AcChR; NPE buffer, 10 mM Na-phosphate, 0.4 M NaCl, 1 mM EDTA, pH 7.4; NRG, pre-immune rabbit Ig.

as the membrane preparations become more enriched in AcChR. Negative staining of Triton-solubilized purified AcChR reveals these same rosettes (3, 9, 32, 38). This correlation of the presence of the rosettes and that of the AcChR, as well as the recent immunospecific labeling of negatively stained membrane fragments with antibodies to AcChR by Klymkowsky and Stroud (18), has led to the identification of this rosette structure as the AcChR molecule. X-ray diffraction studies of AcChR-enriched *T. californica* membrane fragments by Ross et al. (32) have shown that the protein present spanned the membrane, extending 55 Å on one side of the bilayer and 15 Å on the other. Likewise, Rash et al. (30) observed transmembrane staining with $OsO_4/K_3Fe(CN)_6$ of the protein at mammalian neuromuscular junctions and in membrane preparations from *Torpedo oscellata* and correlated this with anti-AcChR antibody binding they observed on one side of these same membranes. In studies of visualization of the binding of ferritin-conjugated anti-AcChR antibodies to *Torpedo* membrane vesicles by electron microscopy of thin sections, Karlin et al. (15) found antibody binding to the outside surface of the vesicles with some labeling on the inside surface which they were unable to distinguish from nonspecific trapping that occurred. Using the same experimental approach, Tarrab-Hazdai et al. (40) observed some binding of anti-AcChR antibodies to both sides of the membrane of some open vesicles as evidence that the receptor spans the membrane.

A major problem in these studies has been in ascertaining the accessibility of both sides of the membrane to the antibody molecules. The recent isolation of intact, outside-out receptor-containing membrane vesicles provides a method for determining which side of the electroplaque synaptic membrane (cytoplasmic or extracellular) corresponds to which side of the isolated membrane vesicles being labeled in such studies (12). We report here the use of such intact vesicles with their extracellular faces exposed for immunospecific labeling of the AcChR molecule. In further studies the vesicles were sheared, exposing their cytoplasmic faces to the immunospecific labeling reagents and providing a well-defined preparation for determining the transmembrane nature of the AcChR.

MATERIALS AND METHODS

Crude membrane fragments were prepared from *T. californica* electroplax by the method of Reed et al. (31). Purified AcChR, solubilized in Triton, was prepared from crude membrane fragments according to Schmidt and Raftery using affinity chromatography (35). Intact, right-side out vesicles enriched in AcChR (0.5–1.5 nmol α-bungarotoxin (α-BuTx) sites per mg of total protein) were made in 10 mM Na-phosphate, 0.4 M NaCl, 1 mM EDTA, pH 7.4 (NPE buffer) (12). Briefly, crude membrane fragments were prepared in isotonic buffer and were then fractionated on a 4–20% sucrose gradient at 195,700 *g* for 1 h. The osmotically intact AcChR-enriched vesicles banded at the top of the gradient. The sidedness of the vesicles was determined by the α-BuTx sites assay described by Hartig and Raftery (12). Intact vesicles were first incubated with an excess of ^{125}I-α-BuTx prepared by the method of Clark et al. (4) and Blanchard et al. (1), in NPE. After 30 min, identical aliquots were diluted into (*a*) 10 mM Na-phosphate, 50 mM NaCl, 0.1% Triton (wash buffer) (total sites sample) and (*b*) wash buffer containing an excess of unlabeled α-BuTx (outside sites sample). After 30-min further incubation, 0.1-ml aliquots were applied to Whatman DE-81 discs and washed for 30 min in wash buffer, with three changes, before counting in a Beckman Gamma-4000 gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). The fraction of outside-out (extracellular side out) vesicles was determined from the ratio [outside sites counts]/[total sites counts].

α-BuTx was purified from the venom of *Bungarus multicinctus* by the method of Clark et al. (4). Triton-solubilized membrane fragment proteins depleted in AcChR (MFTS-R) were prepared by removing the AcChR from Triton-solubilized membrane fragments on an α-BuTx affinity column, and were the gift of T. Claudio.

Protein concentrations were determined by the method of Lowry et al. (22) using bovine serum albumin as the standard. The concentration of α-BuTx sites was determined according to Schmidt and Raftery (36) using DEAE-cellulose filter discs and ^{125}I-α-BuTx. SDS polyacrylamide gel electrophoresis was done according to Laemmli (20), with a 12.5% acrylamide, 0.1% methylene-bis-acrylamide separating gel and a 3% acrylamide, 0.08% bis-acrylamide stacking gel. Immunodiffusion assays were conducted according to Ouchterlony (25).

Preparation of Antibodies

Antibodies to Triton-solubilized, purified AcChR (anti-AcChR) from *T. californica* were raised in New Zealand white rabbits according to Claudio and Raftery (5). The purity of the AcChR was checked on SDS gels before injection, with pure AcChR defined as the complex consisting of only the four polypeptide chains previously described (16, 29). Over a period of 2 yr, six different rabbits were injected with several different AcChR preparations (see Table I); only two of the rabbits (Nos. 5 and 6) received the same AcChR preparation. 0.5–1 mg of AcChR emulsified in Freund's complete adjuvant was injected subcutaneously at multiple sites along the back. 1–2 wk after boosting, all but one of the rabbits displayed the paralysis symptomatic of experimental autoimmune myasthenia gravis (EAMG) (26) and were bled to death. Rabbit No. 2, despite repeated injections, never showed symptoms of the disease; 40 ml of blood was obtained from an ear vein weekly for 2 wk after each challenge of this rabbit. Antibodies to α-BuTx were prepared following the same injection schedule, using 0.1 mg α-BuTx emulsified in Freund's complete adjuvant per injection. The immunoglobulin fraction of antisera (Ig) was prepared by two fractionations with ammonium sulfate (0–33%), and the pellets were dialyzed against phosphate-buffered saline (PBS). This immunoglobulin fraction was clarified by centrifugation at 15,000 *g* for 30 min before dilution into NPE for the labeling experiments. Normal rabbit Ig (NRG) was prepared by ammo-

nium sulfate fractionation of pre-immune serum. Antibody titers were determined by the method of Claudio and Raftery (manuscript in preparation). Triton-solubilized, purified AcChR, bound to ^{125}I-α-BuTx, was incubated with the Ig fraction of the anti-AcChR serum at room temperature. After 1 h, enough *Staphylococcus aureus* to precipitate all the IgG was added and incubated for 30 min. The solution was centrifuged at 15,000 *g* for 5 min, and the pellet was washed in 10 mM Na-phosphate, 0.1% Triton, and counted on a Beckman Gamma-4000 gamma counter.

Hemocyanin-Protein A conjugate (Hcy/A) was prepared by the method of Miller et al. (manuscript in preparation), using glutaraldehyde as the cross-linking reagent. 1 ml of hemocyanin (70 mg/ml), purified by centrifugation and gel filtration from the hemolymph of *Busycon canaliculatum*, and 1 ml of Protein A (Pharmacia Inc., Piscataway, N. J.; 3.6 mg/ml) were conjugated with 0.22 ml of 0.5% glutaraldehyde. After 45 min at room temperature, 0.2 ml of 2 M glycine was added to bind unreacted glutaraldehyde. After 15 min at room temperature, the solution was dialyzed for 24 h at 4°C against PBS, and the Hcy/A conjugate was separated from unconjugated Protein A by passage over a Sepharose 2B column.

Labeling of Intact and Broken Vesicles with Antibodies

INTACT VESICLE LABELING: To attach intact vesicles to cover slips, the glass cover slips were first treated with 1% Alcian Blue (39) for 15 min and washed three times in NPE. 50 μl of intact vesicles (2 mg/ml) were incubated on the cover slip for 15 min, and the excess membranes were rinsed off with NPE. For identification of AcChR antigenic determinants, 35 μl of anti-AcChR Ig from each of the six rabbits (0.5 mg/ml) were then incubated on the cover slip for 30 min at room temperature. The cover slip was then rinsed three times in NPE, and treated with Hcy/A as described below. BuTx-binding sites were visualized by incubation of the cover slip in α-BuTx (0.02 mg/ml) for 45 min before treatment with anti-BuTx Ig and Hcy/A. Control experiments were done by substituting NRG for immune antibodies.

CYTOPLASMIC MEMBRANE LABELING: To expose the cytoplasmic face of the vesicles, a modification of the method of Jacobson and Branton (14) was used. The cover slip was clamped between two pieces of plexiglass with a hole exposing most of the surface of the cover slip. This apparatus was suspended in 1 liter of cold NPE and sonicated in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, N. Y.) at 80 kHz for 5 min. The cover slip was then removed and labeled with antibodies as described above.

HCY/A LABELING OF IMMUNOLABELED VESICLES: After labeling with immune or pre-immune antibodies, the cover slips containing intact or sonicated vesicles were incubated with Hcy/A (1.5 mg/ml in NPE) for 30 min at room temperature and washed three times in NPE.

Preparation of Samples for Electron Microscopy

Immediately after labeling with Hcy/A, samples were fixed in 1% glutaraldehyde in NPE for several hours, followed by fixation in 1% OsO_4 in half-strength NPE for 1 h at 4°C. After dehydration in a graded series of ethanol solutions, the samples were dried at the critical point using CO_2 as the transition fluid in a Polaron apparatus (Polaron Instruments Inc., Line Lexington, Pa.). The cover slips were shadowed at a 45° angle with platinum/palladium followed by coating with carbon at a 90° angle in a vacuum evaporator. Replicas were then separated from the glass cover slip by etching with 48% hydrofluoric acid, and the membrane fragments were removed with twice-filtered household bleach. After rinsing with water, the replicas were deposited on copper grids and examined in a Philips 201 electron microscope.

RESULTS

The intact vesicles described here were from 94 to 100% extracellular side-out, i.e., 94–100% of the total α-BuTx sites were exposed on the outside surface of the vesicles, as determined by the α-BuTx binding assay described in Materials and Methods. When examined at the electron microscope level, replicas of these preparations consisted of spherical vesicles ranging in diameter from 0.1 to 1 μm (see Fig. 1 *a*). Upon sonication for 5 min, the vesicles appeared as flat circles with a rough texture, often with a thickening at the perimeter attributable to edges not attached to the Alcian-blue coating (Fig. 1 *e*). A time-course of the effects

TABLE I

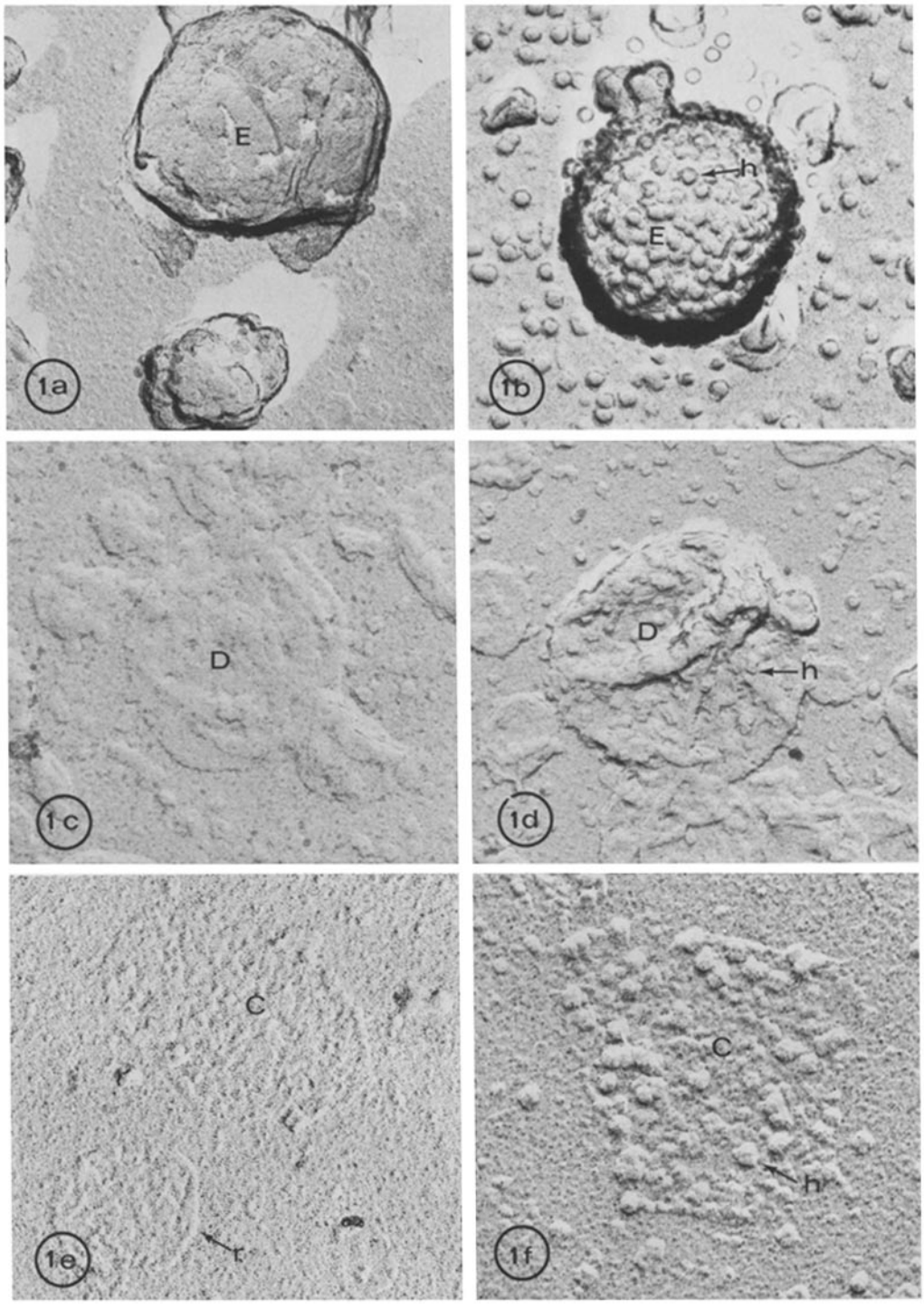
Characterization of Anti-AcChR Antisera

		Titers*		Ouchterlony reactions‡			EM studies with Hcy/A	
Rabbit No.	Contracted EAMG	nmol α-BuTx sites	pmol AcChR	AcChR	MFTS	MFTS-R	Extracellular labeling	Cytoplasmic labeling
1	yes	0.32	2.00	++++	++++	—	yes	no
2	no	0.51	3.00	++++	++++	++	yes	no
3	yes	0.65	4.06	++++	++++	−	yes	no
4	yes	0.54	3.37	++++	++++	+	yes	no
5§	yes	1.95	12.17	++++	++++	−	yes	yes
6§	yes	2.35	14.16	++++	++++	−	yes	no

* Titers are given per milliliter of serum.

‡ Ouchterlony results are as judged visually, with ++++ indicating a very strong reaction, + a very weak reaction, and — no reaction.

§ These two rabbits were injected with the same preparation of AcChR.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of sonication showed that after 2 min most vesicles maintained intact their spherical shape, while by 5 min ~95% of the vesicles had been sheared, leaving their inside faces exposed. At times longer than 10 min vesicles were frequently totally removed from the Alcian blue, and after 30 min the Alcian blue began to pucker and detach. Occasionally, vesicles could be found which had collapsed during sonication and were not sheared off. Such collapsed vesicles could be readily distinguished from sheared vesicles in both labeled and unlabeled preparations by the smoother and more three-dimensional appearance of the former (see Fig. 1).

The binding of anti-AcChR and anti-BuTx Ig to intact and sheared vesicles is summarized in Tables II and III. That the intact vesicles were oriented mainly so that their extracellular, α-BuTx-binding side faced outward was verified at the electron microscope level by the binding of α-BuTx and anti-BuTx Ig, visualized with the Hcy/A marker. As can be seen in Fig. 2*a*, the outsides of several vesicles treated with α-BuTx and anti-BuTx Ig, visualized with Hcy/A, were coated with the Hcy/A markers. Upon sonication, the inside surfaces of the vesicles were exposed but no α-BuTx binding could be discerned (Fig. 2*b*). Control experiments in which vesicles were treated with NRG and Hcy/A showed no labeling on either the intact or the sonicated vesicles (Fig. 3). With all six anti-AcChR Ig preparations, the extracellular faces of the membranes were labeled (Fig. 4*a*). With both anti-BuTx and anti-AcChR Ig's, a high proportion (70–80%) of the larger intact vesicles (~0.5 μm in diameter and larger) were heavily labeled, while most of the smaller vesicles and some of the larger ones were not labeled at all with either antibody.

When the sonicated vesicles were treated with anti-AcChR Ig, there was a dramatic difference in labeling from one antibody preparation to the next. With anti-AcChR Ig's from rabbits Nos. 1, 2, 3, 4, and 6, there was little if any labeling on the insides of the vesicles (Fig. 4*b*). Occasionally, inside labeling of the vesicles was observed (Fig. 4*c*), but it was usually sparse and could be correlated with an increase in the background labeling of the Alcian blue, suggesting that insufficient washing may have caused nonspecific disposition of Hcy/A on the vesicle surface. In other cases (Fig. 4*d*), label was observed only around free edges of the vesicle which had curled over and faced upward, exposing a narrow rim of external AcChR sites to the anti-AcChR Ig solution.

With anti-AcChR Ig from rabbit No. 5, sonicated vesicles were heavily labeled (Fig. 4*e*). The density of Hcy/A markers on the vesicles was up to 15 times higher than that on the Alcian-blue background, an increase comparable to that observed in the labeling of intact vesicles with anti-AcChR Ig (compare Fig. 4*a* and *e*). As in the labeling of intact vesicles, it was mainly the larger sonicated vesicles that were labeled with anti-AcChR Ig from rabbit No. 5; most of the smaller vesicles and some of the larger ones did not label at all (Fig. 4*f*).

The labeling results are summarized in Tables II and III. All vesicles in large fields (each containing ~200 vesicles) of intact or sheared vesicles treated with each of the anti-AcChR preparations, α-BuTx + anti-BuTx, or NRG were examined and the percentage of vesicles labeled with Hcy/A

FIGURE 1 (*a*) Two intact *Torpedo* electroplaque membrane vesicles, with the extracellular side (*E*) of the synaptic membrane exposed. The vesicles are smooth and spherical. × 60,000. (*b*) An intact vesicle, extracellular side (*E*) exposed, labeled with anti-AcChR and Hcy/A. The spherical shape of the vesicle can be distinguished beneath the hemocyanin markers (*h*). × 50,000. (*c*) A deflated membrane vesicle (*D*), with its extracellular side exposed, appears as a smooth but flat circle. × 43,000. (*d*) A deflated vesicle (*D*), labeled with anti-AcChR and Hcy/A. The smooth surface of the vesicle can be seen beneath the hemocyanin markers (*h*). (*e*) Membrane vesicles after 5-min sonication. The vesicles have been sheared, leaving the rough cytoplasmic face (*C*) of the membrane exposed. A thickening around the rim of the vesicle (*r*) can be attributed to membrane not attached to the Alcian blue. These vesicles can be readily distinguished from deflated vesicles (Fig. 1*c*) by the roughness of the cytoplasmic face. × 68,000. (*f*) A sheared membrane vesicle after 5-min sonication, labeled with anti-AcChR from rabbit No. 5 and Hcy/A. The rough, cytoplasmic face of the membrane (*C*) can be identified beneath the hemocyanin markers (*h*). These sheared, labeled vesicles can be easily distinguished from intact, labeled vesicles and deflated, labeled vesicles, as can be seen by a comparison of *b*, *d*, and *f*. × 50,000.

TABLE II

Antibody Labeling of Vesicles in a Single Field, According to Size

Vesicle diameter		≥5,000 Å		3,000–5,000 Å		1,000–3,000 Å		≤1,000 Å	
Antibody preparation	Vesicle preparation	No. labeled/total	% Labeled	No. labeled/total	% Labeled	No. labeled/total	% Labeled	No. labeled/total	% Labeled
Anti-AcChR No. 1	Intact	5/7	71	11/36	31	3/152	2	0/72	0
	sheared	0/3	0	1/6	17	0/30	0	0/22	0
Anti-AcChR No. 2	Intact	7/13	54	14/40	35	3/106	3	0/66	0
	sheared	0/3	0	0/10	0	0/51	0	0/120	0
Anti-AcChR No. 3	Intact	7/8	88	10/42	24	6/147	4	0/73	0
	sheared	0/2	0	0/10	0	0/22	0	0/92	0
Anti-AcChR No. 4	Intact	8/11	73	9/31	29	6/116	3	0/89	0
	sheared	0/4	0	1/12	8	1/11	9	0/43	0
Anti-AcChR No. 5	Intact	5/7	71	7/24	29	6/163	2	0/70	0
	sheared	5/6	83	10/38	26	3/45	7	0/78	0
Anti-AcChR No. 6	Intact	4/5	80	10/40	25	5/152	3	0/87	0
	sheared	0/3	0	1/34	3	3/47	6	0/49	0
Anti-BuTx	Intact	4/5	80	8/36	22	2/108	2	0/95	0
	sheared	0/2	0	0/21	0	0/26	0	0/92	0
NRG	Intact	0/10	0	0/52	0	0/104	0	0/83	0
	sheared	0/8	0	0/12	0	0/27	0	0/17	0

TABLE II, SUMMARY

(A) Extracellular surface labeling (% labeled)					(B) Cytoplasmic surface labeling (% labeled)				
Antibody \ Vesicle diameter	≥5000 Å	3,000–5,000 Å	1,000–3,000 Å	≤1,000 Å	Antibody \ Vesicle diameter	≥5,000 Å	3,000–5,000 Å	1,000–3,000 Å	≤1,000 Å
Anti-AcChR No. 1–6	73 ± 9	29 ± 4	3 ± 0.8	0	Anti-AcChR Nos. 1–4,6	0	6 ± 7	3 ± 4	0
Anti-BuTx	80 ± 10	22 ± 3	2 ± 0.5	0	Anti-AcChR No. 5	83 ± 10	26 ± 4	7 ± 7	0
NRG	0	0	0	0	Anti-BuTx	0	0	0	0
					NRG	0	0	0	0

All vesicles in a single field were counted for each antibody category and the percentage of labeled vesicles for each size range determined. The results are summarized in the lower half of the table.

TABLE III

Antibody Labeling of Intact and Sheared Vesicles

Vesicle diameter	≥5,000 Å		3,000–5,000 Å	
Antibody	No. labeled/total	% Labeled	No. labeled/total	% Labeled
(A) Extracellular surface labeling				
Anti-AcChR Nos. 1–6	56/73	77 ± 9	54/189	29 ± 4
Anti-BuTx	35/49	71 ± 9	11/49	22 ± 3
NRG	0/52	0	0/52	0
(B) Cytoplasmic surface labeling				
Anti-AcChR Nos. 1–4,6	2/58	3 ± 3	3/78	4 ± 3
Anti-AcChr No. 5	39/50	78 ± 10	13/51	25 ± 3
Anti-BuTx	2/52	4 ± 3	1/57	2 ± 2
NRG	0/51	0	0/42	0

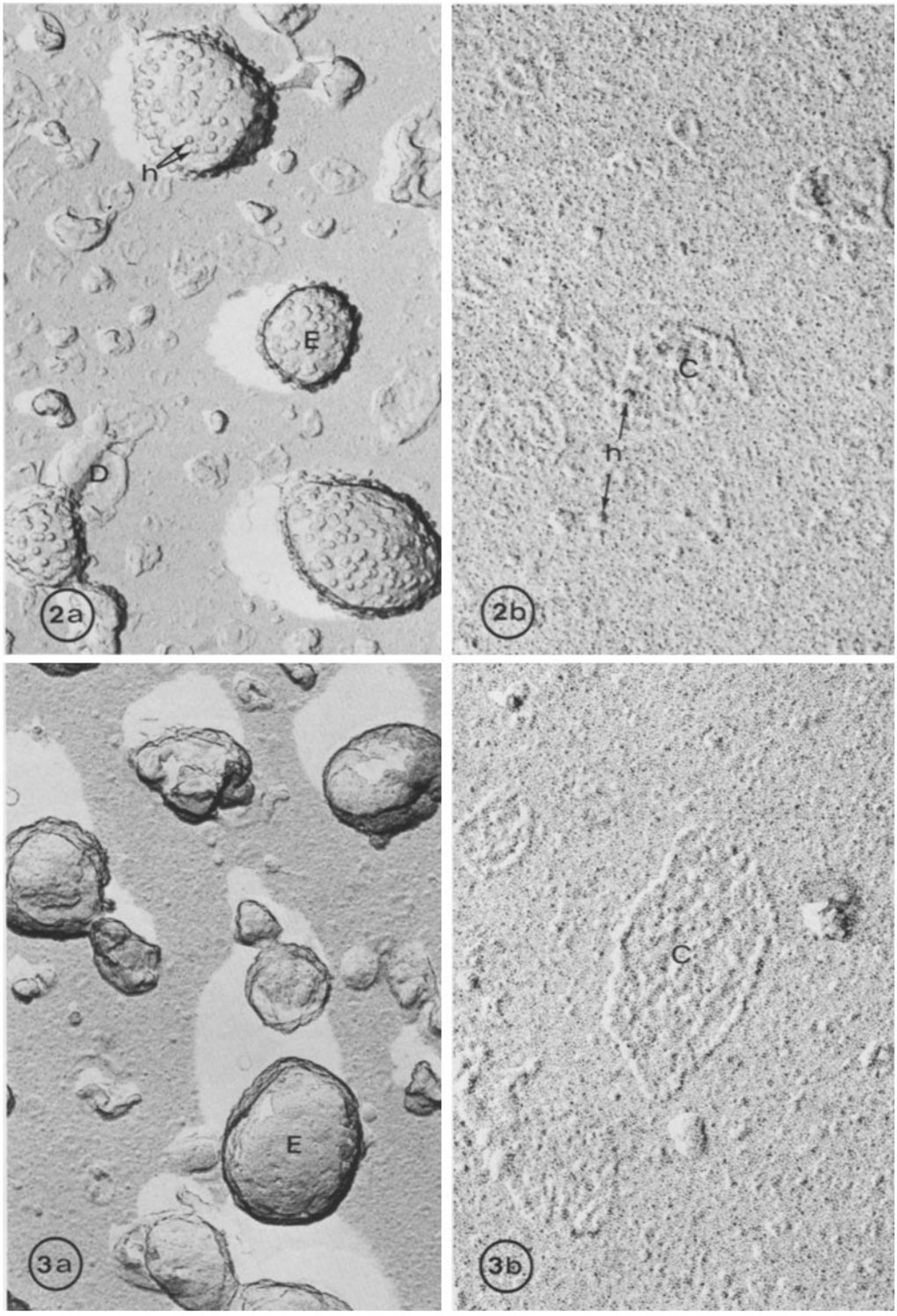
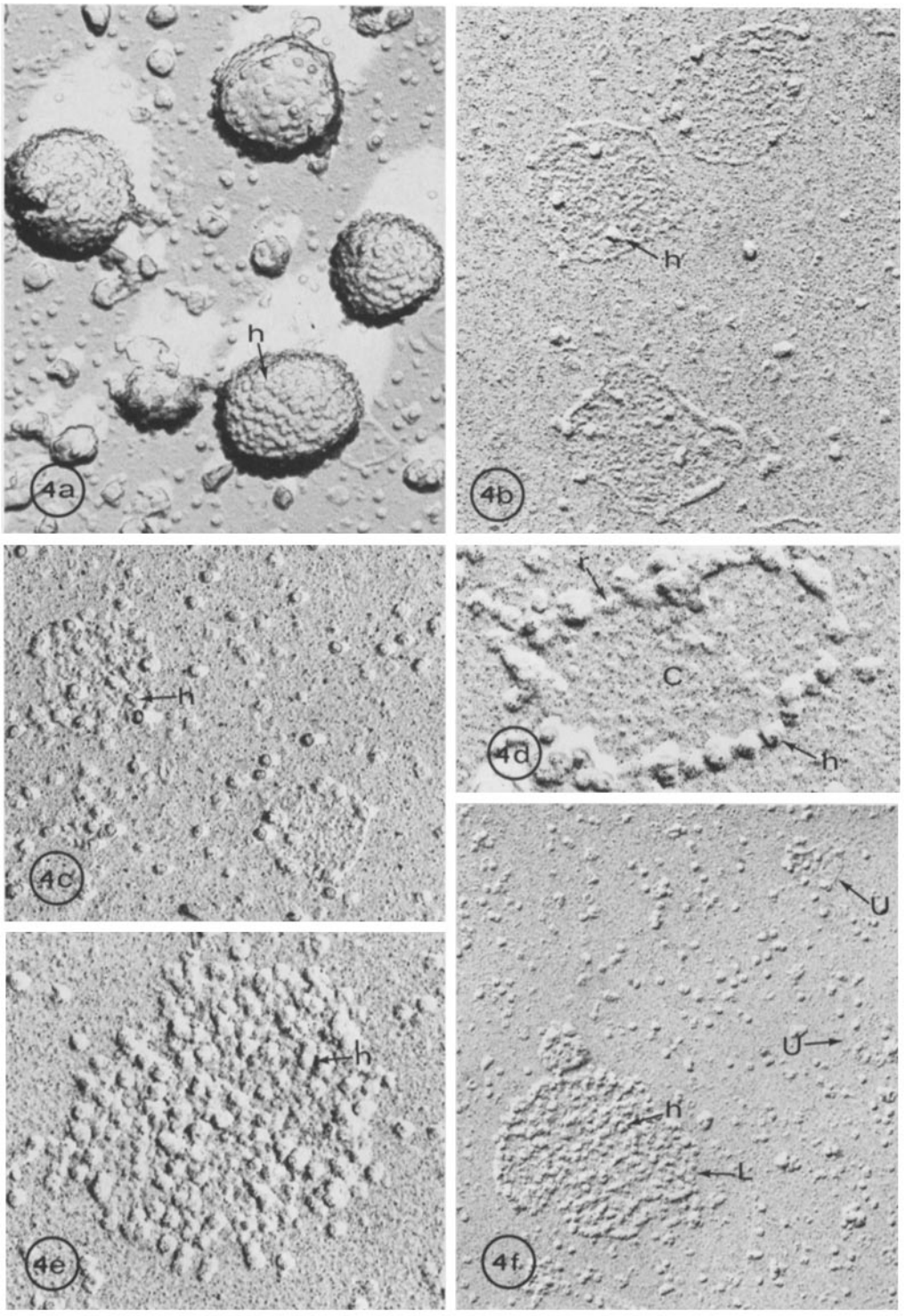


FIGURE 2 Vesicles treated with α-BuTx followed by anti-BuTx and Hcy/A. (*a*) Hemocyanin molecules (*h*) can be seen as round or square particles on the external surface (*E*) of the larger intact vesicles. The small vesicles are not labeled and are probably lipid vesicles. A deflated vesicle (*D*) can be identified by its distinctive shape and smoothness. × 31,000. (*b*) After sonication, the vesicles are sheared, exposing their cytoplasmic sides (*C*). Hemocyanin molecules (*h*) appear in the background, but only one can be detected at the edge of a vesicle. × 49,500.

FIGURE 3 Control experiment in which vesicles were treated with pre-immune rabbit Ig and Hcy/A. (*a*) With intact vesicles there was no labeling of the extracellular side of the membrane (*E*). × 50,000. (*b*) After sonication, the cytoplasmic side of the membrane (*C*) was exposed and did not label. × 81,000.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markers for each of four size ranges (diameters of ≥5,000; 3,000–5,000; 1,000–3,000; and ≤1,000 Å) was determined. As is clear from Table II, the AcChR-containing vesicles are essentially all larger than 3,000 Å in diameter, with 70–80% of the intact vesicles larger than 5,000 Å and 20–30% of those in the 3,000–5,000 Å range containing AcChR, as determined by Hcy/A visualization of both anti-AcChR and α-BuTx + anti-BuTx binding. To provide a better statistical sample from which to determine whether anti-AcChR from rabbit No. 5 did indeed consistently label the insides of AcChR-containing vesicles, 40 or more vesicles with diameters of 3,000–5,000 Å and 40 or more with diameters ≥ 5,000 Å were examined for Hcy/A labeling after treatment with anti-AcChR, α-BuTx + anti-BuTx, or pre-immune Ig. Anti-AcChR from rabbits Nos. 1–4 and 6 and anti-BuTx behaved the same way, again labeling 70–80% of the largest vesicles and 20–30% of the 3,000- to 5,000-Å vesicles on the extracellular side of the membrane and essentially none on the cytoplasmic side. This larger sampling also confirmed the result indicated in the smaller sampling in Table II: anti-AcChR from rabbit No. 5 labeled the cytoplasmic side of the membranes on 78% of the ≥5,000-Å vesicles and 25% of the 3,000- to 5,000-Å vesicles, the same amount of labeling observed on the extracellular sides of these vesicles with α-BuTx + anti-BuTx and with all six anti-AcChR preparations (including that from rabbit No. 5). Pre-immune Ig labeled on neither the insides nor the outsides of vesicles in any size range.

Ouchterlony immunodiffusion assays of each anti-AcChR Ig preparation against purified AcChR, Triton-solubilized membrane fragments (MFTS), and MFTS-R showed a strong reaction of all anti-AcChR Ig against AcChR and against MFTS (see Fig. 5). Only one preparation (from rabbit No. 2) reacted moderately with the MFTS-R (see Table I). With one other anti-AcChR Ig preparation, a barely discernible reaction was recorded. With the other four anti-AcChR Ig preparations (including that from rabbit No. 5), no reaction with the MFTS-R was observed.

DISCUSSION

The experimental evidence presented here indicates that the AcChR, composed of four subunits with mol wt of 40,000, 50,000, 60,000, and 65,000 (16, 28, 29), is a transmembrane protein. The method of immunospecific labeling used permits complete exposure of both the extracellular and the cytoplasmic faces of the membrane to the antibody solution. These faces could be clearly identified as the extracellular face of the synapse corresponding to the outside face of the membrane vesicle and the cytoplasmic face to the inside of the vesicle by Hcy/A visualization of the binding of α-BuTx and anti-BuTx. Hemocyanin has proven to be a highly visible marker in TEM studies of smooth cellular or membrane surfaces (2, 37), and the specificity of Hcy/A for IgG, already established by Miller et al. (manuscript in preparation), is here shown again by the lack of labeling with NRG. The use of osmotically intact vesicles permitted the use of a ^{125}I-α-BuTx binding assay to confirm that most of the vesicles were indeed "right-side out," i.e., most of the α-BuTx sites were exposed on the outside surface of the vesicles, in agreement with the observations of Hartig and Raftery (12).

In the labeling of intact vesicles with anti-AcChR Ig, the results obtained with antisera from different rabbits were in excellent agreement. An-

FIGURE 4 Vesicles treated with anti-AcChR followed by Hcy/A. (*a*) Intact vesicles treated with any of the six anti-AcChR preparations were labeled with hemocyanin (*h*) on their external surfaces. As in Fig. 2, most of the larger vesicles were labeled, while the smaller ones were not. × 31,000. (*b–d*) Vesicles after sonication, treated with anti-AcChR from rabbit No. 1, 2, 3, 4, or 6. (*b*) Few hemocyanin molecules (*h*) can be seen on the cytoplasmic side of the membrane or on the background. × 37,000. (*c*) An increase in the hemocyanin (*h*) labeling of the cytoplasmic membranes corresponds to an increase in the hemocyanin in the background. × 43,000. (*d*) The cytoplasmic face (*C*) of the sheared vesicle can be seen with thickening around the free edges (*r*). The entire rim is labeled with hemocyanin molecules (*h*), with none in the center. × 66,000. (*e* and *f*) Vesicles after sonication, treated with anti-AcChR from rabbit No. 5. (*e*) A close-up of one vesicle shows the characteristic rough cytoplasmic side of the membrane heavily labeled with hemocyanin (*h*). × 47,000. (*f*) A view of a larger field shows sheared vesicles with their cytoplasmic sides exposed. (*L*) A large vesicle is labeled with hemocyanin (*h*), while several smaller vesicles are unlabeled (*U*). × 22,000.

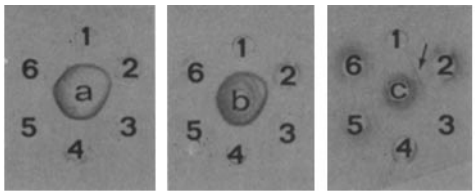

FIGURE 5 Ouchterlony immunodiffusion assays. The numbers *1-6* indicate the wells which were filled with anti-AcChR Ig from rabbits 1–6. The antigens in the center wells were (*a*) AcChR, (*b*) MFTS, and (*c*) MFTS-R. The arrow points out the faint cross-reaction observed between MFTS-R and Ig from rabbit No. 2.

tisera from all rabbits showed equal labeling of the outside (extracellular) faces of intact vesicles. The observation that the same proportion of vesicles of each size range was labeled with both anti-BuTx and anti-AcChR Ig is indicative of the specificity of the antibodies for the AcChR since it indicates that the same vesicles were labeled with both reagents. The differences in labeling from one antibody preparation to the next became apparent only in the labeling of the cytoplasmic faces of sonicated vesicles, where anti-BuTx and five of the six preparations of anti-AcChR showed little or no labeling and one showed labeling comparable to that seen on the extracellular face of the membranes.

That the labeling seen with anti-AcChR from rabbit No. 5 was specific labeling of the AcChR on the cytoplasmic face of the membrane was the conclusion drawn from several lines of evidence. (*a*) The AcChR injected into the rabbit producing these antibodies was highly purified as judged by its SDS gel electrophoresis profile, having a gel pattern consistent with that of pure *T. californica* AcChR (16, 28, 29). Furthermore, the same AcChR preparation was injected at the same time into another rabbit (No. 6) whose antisera were also used in these studies. The antibodies from rabbit No. 6 did not react with the cytoplasmic sides of the vesicles, ruling out the possibility that there was simply a variation in the AcChR preparation used to immunize the rabbit which caused this labeling of the cytoplasmic face. (*b*) The immunodiffusion results indicate that it is in all likelihood not a contaminant in the AcChR injected into the rabbit that causes labeling of the cytoplasmic face with the antibodies from rabbit No. 5. In the interaction between the various anti-AcChR Ig preparations and MFTS-R, there was no discernible reaction with immunoglobulins from rabbit No. 5. There was, however, a very faint reaction with the antisera from rabbit No. 2 and a barely discernible reaction with antisera from rabbit No. 4, neither of which labeled the cytoplasmic face. (*c*) All labeling experiments were repeated three times with three different intact vesicle preparations, yielding the same results. Therefore, the labeling of the cytoplasmic face of the membrane by this anti-AcChR preparation was a real phenomenon and not an artifact caused by insufficient washing of one sample. (*d*) Finally, the statistics of labeling given in Tables II and III indicate that the AcChR-containing vesicles can be identified by their large size. It is these large vesicles that label with both anti-AcChR (77%) and anti-BuTx (71%) on the extracellular face and which also label with this anti-AcChR preparation (78%) on the cytoplasmic face. This statistical agreement also holds for vesicles in the 3,000- to 5,000-Å range, 20–30% of which contain AcChR. That none of the vesicles labeled with pre-immune Ig on either side nor with α-BuTx + anti-BuTx on the cytoplasmic side also indicates that the labeling observed is specific for the given antibody-antigen interaction.

It is not unusual that different rabbits injected with the same antigen would produce antibodies of differing specificities; these experimental rabbits are outbred population and their immune responses are not homogeneous. Such differences in the specificities of rabbit antibodies have been found in studies of the functional effects of anti-AcChR antibodies (15, 33) and of anti-Na^+,K^+-ATPase antibodies (19). That only one out of six rabbits displayed an immune response to antigens on the inside face of the membrane indicates either that the portion of the receptor exposed on this side is not very antigenic and/or that only a small portion of the molecule is exposed on the cytoplasmic face. This concurs with the findings of Ross et al. (32), who determined that protein protruded only 15 ± 5 Å on one side of the membrane of AcChR-enriched preparations while extending 55 ± 5 Å from the other side.

In thin-section electron micrographs of the labeling of membrane-fragments in solution with ferritin-conjugated anti-AcChR antibodies, Tarrab-Hazdai et al. (40) saw some labeling of both sides of open vesicles, while Karlin et al. (15) saw some inside labeling, but were unable to distinguish it from nonspecific sticking of ferritin. This difference may also have resulted from differing specificities of the anti-AcChR antibody prepara-

tions used. In the labeling of whole vesicles with antibody molecules, the insides are often not accessible to the labeling reagents (40; C. D. Strader, unpublished observations) and there can be nonspecific trapping of labels inside the vesicles. Preliminary observations both by electron microscopy (C. D. Strader, unpublished observations) and by flux measurements (H.-P. Moore and P. Hartig, unpublished observations) indicate that *Torpedo* membrane vesicles reseal after osmotic shock, thus complicating this experimental approach. The labeling method described here avoids any ambiguities caused by inaccessibility or by trapping of label inside the vesicles, since only the extracellular side or only the cytopasmic side of the membrane is completely exposed to the labeling solution.

The AcChR consists of four subunits of mol wt 40,000, 50,000, 60,000, and 65,000 daltons (16, 28, 29). By lactoperoxidase-catalyzed iodination of the outsides of intact vesicles, Hartig and Raftery (manuscript in preparation) have shown that portions of all four of the AcChR subunits are exposed on the extracellular face of the synaptic membrane. Lindstrom et al. (21) have shown that antibodies to each of the AcChR subunits cross-react with rat muscle receptors in vivo, causing a decrease in muscle AcChR content. Hence, each of these four polypeptide chains must contain at least some antigenic determinants exposed on the extracellular face of the membrane.

Attempts to label the membrane vesicles with antibodies to individual AcChR subunits have been unsuccessful. This is probably due to the failure of such antibodies (prepared against the denatured individual subunits isolated from preparative SDS polyacrylamide gels) to react with native, membrane-bound AcChR as observed with electron microscopy. Therefore, it was not possible to determine by this method which of the subunits are exposed on the extracellular side and which on the cytoplasmic side of the membrane.

In conclusion, we have developed an unambiguous method for immunospecific labeling of the extracellular face or of the cytoplasmic face of membrane vesicles enriched in AcChR. The AcChR is a complex molecule consisting of four polypeptide subunits which remain together as a complex in the presence of Triton (35) and cholate (Elliott et al., manuscript in preparation) and which dissociate only when denatured in SDS (29). Antibodies to the AcChR containing only these four subunits were prepared and used to label a preparation of vesicles (12) in which 94–100% of the α-BuTx-binding sites were on the outside surface of the membrane. Thus, in these vesicles, almost all of the AcChR molecules were at least partially exposed to the outside surface of the vesicles (corresponding to the extracellular face of the postsynaptic membrane). All preparations of anti-AcChR antibodies were found to bind to the outer surface of the vesicles, and one preparation also bound to the inner surface of the vesicles. Therefore, one or more of the four polypeptide subunits of the AcChR used as the antigen for production of these antibodies is exposed on the extracellular face of the membrane, and one or more is exposed on the cytoplasmic face of the membrane. Thus, it may be concluded from this evidence that the AcChR is a transmembrane protein, with many antigenic determinants on the extracellular face of the synaptic membrane and with few of the antigenic determinants protruding on the cytoplasmic face of the membrane.

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