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# Localization of acetylcholine receptor in excitable membrane from the electric organ of *Torpedo*: Evidence for exposure of receptor antigenic sites on both sides of the membrane

(nicotinic acetylcholine receptor/receptor-rich membrane fragments/immunoferritin labeling/electron microscopy/transmembrane protein)

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ABSTRACT **Nicotinic acetylcholine receptor was localized in a receptor-rich membrane preparation from the electric organ of *Torpedo californica* by applying an immunoferritin technique. The membrane preparation was incubated with $(Fab')_2$ fragments derived from specific rabbit antibodies against the purified acetylcholine receptor and subsequently with ferritin-conjugated goat antiserum to rabbit immunoglobulin. More than 50% of the vesicles were found to be labeled with ferritin while the rest remained unlabeled. Ferritin labeling on both sides of the membrane was evident in open membrane vesicles, whereas in closed vesicles the labeling was confined to the outer surface due to the inability of the tracer to penetrate the membrane. These data suggest that antigenic sites of the receptor molecule are exposed on both sides of the excitable membrane, and that acetylcholine receptor may be a transmembrane protein.**

The electric organ of *Torpedo* is rich in nicotinic acetylcholine receptor (AcChoR) and has been extensively used for the isolation, purification, and characterization of the receptor molecule (for reviews see refs. 1–4).

Isolation of AcChoR-rich membrane fractions (5) enables a detailed analysis of the pharmacological and physiological properties of the nicotinic AcChoR in the excitable membrane (6–9). Furthermore, such preparations of membrane vesicles appear to be suitable for ultrastructural analysis of the organization of the receptor molecules in the membrane. The use of radiolabeled $\alpha$-neurotoxins, which bind specifically to the nicotinic AcChoR, gave some qualitative and quantitative insight into the distribution of AcChoR molecules in electrogenic tissue and in isolated membranes derived from it (8, 10). Such studies pointed to the preferential confinement of the receptor to the postsynaptic membrane and gave indications on the density of the receptor in its native, membranous environment. Freeze-fracture studies of the *Torpedo* electric organ revealed packed arrays of intramembrane particles of 80–90 Å diameter in the postsynaptic membrane (11, 12). Ultrastructural studies by freeze fracturing or negative staining of receptor-rich membrane fractions isolated from this tissue demonstrated an organized hexagonal array of closely packed particles in a high portion of the membrane vesicles (9, 11, 13, 14). The dimensions of these particles (80–90 Å with a central "hole" of 15 Å diameter) closely resemble the values obtained for pure AcChoR (15). The density of these structures was about 12,000–15,000 per $\mu m^2$ (13), in agreement with the density of receptor in such preparations, calculated from the specific binding of radiolabeled neurotoxins (10).

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It has been suggested (3) that receptor molecules may be exposed at both the extracellular and cytoplasmic faces of the membrane, but no direct evidence for such orientation is available. In the present study we have localized AcChoR in a preparation of receptor-rich membrane vesicles from *Torpedo californica* electric organ by specific immunoferritin staining, using $(Fab')_2$ fragments of antibodies against highly purified AcChoR. Our data suggest that AcChoR is a transmembrane protein exposed on both faces of the membrane.

## MATERIALS AND METHODS

**Preparation of AcChoR-Rich Membranes from *Torpedo californica*.** Frozen electric organs of *Torpedo californica* (Pacific Bio-marine, Venice, CA; 50 g) were used for the preparation of membranes. The first steps, which included homogenization, sonication, and ultracentrifugation through a sucrose gradient, were performed according to Cohen *et al.* (5). The receptor-rich fractions were pooled, diluted 10-fold with phosphate-buffered saline ($P_i$/NaCl), and sedimented at 16,700 × *g* (4°, 10 min). Membranes from 50 g of original tissue were suspended in 5 ml of $P_i$/NaCl and incubated with 1 mg of DNase (Miles, 2400 units/mg). After 20 min at room temperature, the membranes were sedimented (16,700 × *g*, 10 min, 4°) and washed twice with $P_i$/NaCl. This additional step seems to greatly improve the quality of the membrane preparation by eliminating electron-dense deposits that bind ferritin nonspecifically. No decrease in receptor binding sites of the membrane preparation was detected following the DNase treatment. $^{125}$I-Labeled $\alpha$-bungarotoxin binding activity of the resulting receptor-rich membrane preparation was about 5000 pmol/mg of protein.

**Immunochemical Reagents.** Anti-AcChoR antibodies were evoked in rabbits by immunization with highly purified AcChoR from *Torpedo californica*. The receptor used for immunization, which was purified by affinity chromatography on a Sepharose-*Naja naja siamensis* neurotoxin column, had a specific activity of 10,000 pmol of toxin per mg of protein, showed a single 9S peak on sucrose gradient centrifugation and by analytical ultracentrifugation, and gave one precipitation line with anti-AcChoR serum in both immunodiffusion and immunoelectrophoresis. The detailed procedure employed for the purification of the receptor, the assessment of its purity, and the immunization scheme have been previously described (16). The IgG fraction was isolated by ammonium sulfate fractionation and ion exchange chromatography on DEAE-cellulose (17). $(Fab')_2$ fragment of anti-AcChoR was prepared by pepsin

Abbreviations: AcChoR, acetylcholine receptor; $P_i$/NaCl, phosphate-buffered saline.

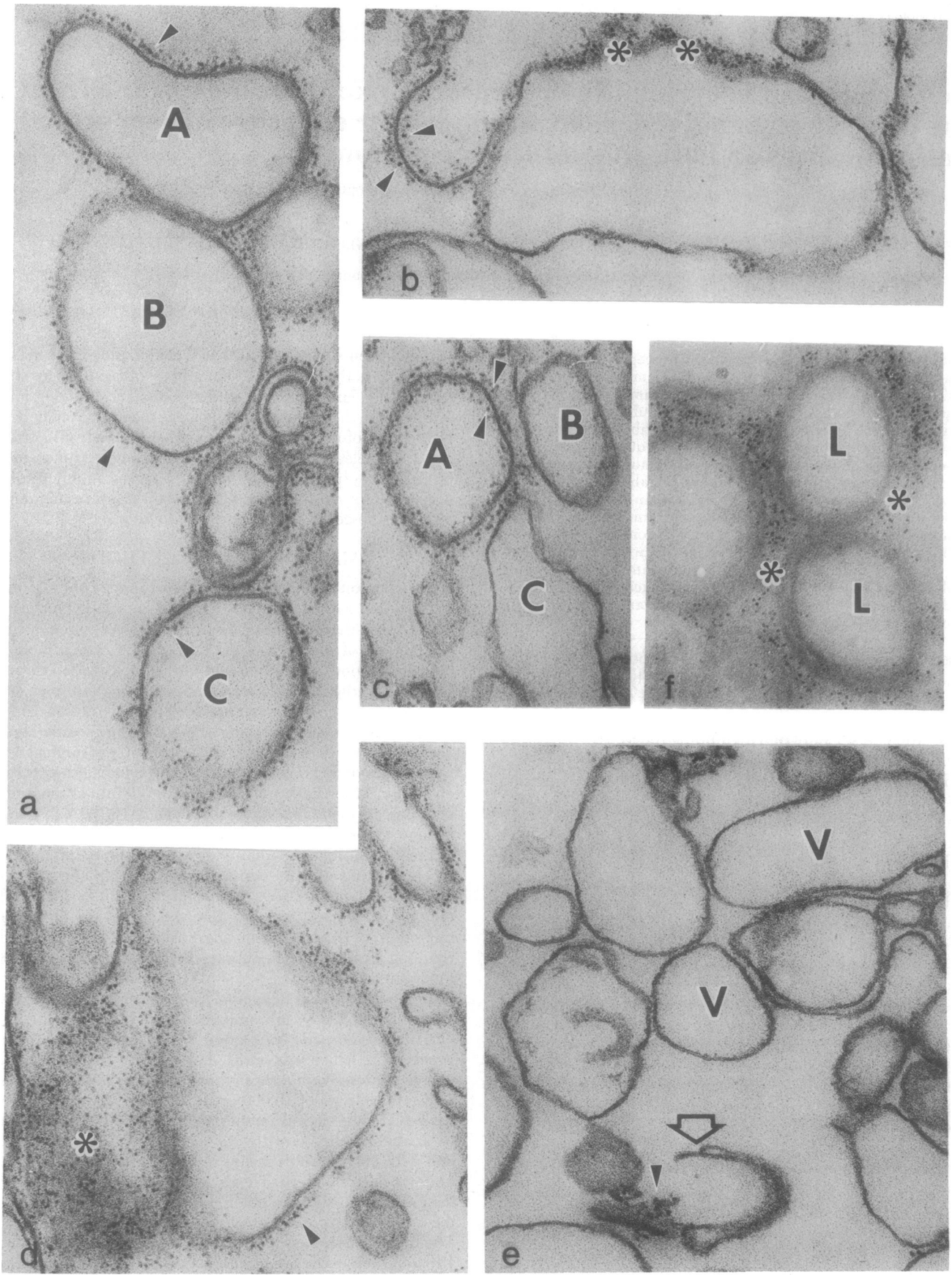


FIG. 1. Thin sections of receptor-rich membrane vesicles from *Torpedo californica* electric organ tissue. The sections shown in *a–d* were incubated with $(Fab')_2$ fragments of rabbit anti-AcChoR IgG and subsequently with ferritin-conjugated goat anti-rabbit IgG; sections were stained for 20 sec with lead citrate. (*a*) Ferritin molecules (arrowheads) cover the outer surface of closed membrane profiles (A and B) in a rather uniform layer, while in vesicle C, which seems to be unsealed, several ferritin molecules (arrowhead) can be seen to label the membrane from

digestion (18). Anti-AcChoR IgG fraction was incubated with 140 μg of pepsin (Worthington Biochemical Corp., 2500 units/mg) in 0.1 M acetate buffer, pH 4.5. After 16 hr at 37°, the reaction mixture was brought to pH 7.0 and dialyzed against $P_i$/NaCl. Gel filtration of the digest on a Sephadex G-100 column showed one protein peak with a molecular weight of about 100,000. The activity of the resulting $(Fab')_2$ fragment was verified by its capacity to bind $^{125}I$-labeled AcChoR and to inhibit binding of $^{125}I$-labeled α-bungarotoxin to AcChoR (16). $(Fab')_2$ fragment of IgG against a nonrelevant antigen (*Naja naja siamensis* toxin) was prepared by the same procedure and used for control experiments.

Ferritin-conjugated goat anti-rabbit IgG was purchased from Miles–Yeda (Rehovot, Israel). The concentration of the stock solution was 18 mg of IgG and 27 mg of ferritin per ml. Prior to staining, conjugate solutions were centrifuged at 20,000 × *g* for 20 min to remove aggregates.

**Immunoferritin Staining of AcChoR-Rich Membranes.** Immunochemical staining of *Torpedo californica* membranes was performed using an indirect immunoferritin technique; membranes corresponding to 3–5 g of original tissue were pelleted in micro-test tubes (Eppendorff, W. Germany) and suspended in 0.3 ml of anti-AcChoR $(Fab')_2$ (1 mg/ml). After 30 min. at 25°, the membranes were washed three times with 1 ml of $P_i$/NaCl and suspended in 0.3 ml of ferritin-conjugated goat anti-rabbit IgG (1.8 mg/ml with respect to the IgG content in the conjugate). The suspension was further incubated at 25° for 30 min, after which the membranes were washed three times with $P_i$/NaCl.

In control experiments, membranes were incubated with the nonrelevant $(Fab')_2$ instead of the anti-AcChoR $(Fab')_2$ and processed subsequently as described above.

**Electron Microscopy.** Fresh pellets of material were resuspended in small volumes (0.1–0.2 ml) of $P_i$/NaCl and fixed for 2 hr at 24° in 3% (wt/vol) glutaraldehyde/1% (wt/vol) formaldehyde/0.1 M sodium cacodylate, pH 7.4 (19). To examine whether the ferritin-IgG conjugate can penetrate into the lumen of the membrane vesicles, membrane samples were incubated with the conjugate (9 mg/ml with respect to the IgG content) for 30 min at 25° without prior incubation with any $(Fab')_2$ solution and fixation was carried out as above without washing the excess conjugate. Fixed suspensions were centrifuged in polyethylene tubes (Microfuge 152, Beckman Instruments Inc., Fullerton, CA) at 10,000 × *g* for 15 min. The pellets were postfixed for 2 hr at 4° with 1% osmium tetroxide in the same buffer and stained *en bloc* for 1 hr at 24° with 0.5% uranyl acetate in Veronal/acetate buffer, pH ~5 (20). The pellets were dehydrated in ethanol and propylene oxide and embedded in Epon (21). Thin sections were cut parallel to the direction of sedimentation so as to include all layers of the pellet. Thin sections were examined [either without staining or after staining for 20 sec with lead citrate (0.4%) in 0.1 M NaOH] in a JEOL 100 B electron microscope.

## RESULTS

The receptor-rich membrane preparation described in this study contained mainly smooth membrane vesicles ranging in size from about 0.1 to 1.0 μm. Cell debris and amorphous material that were observed before DNase treatment were significantly reduced after this treatment. Most of the vesicles exhibited a closed profile, but occasionally open vesicles could be seen in the preparation (Fig. 1 *a*, *b*, and *e*). Nevertheless, a clear unit membrane with a thickness of about 70 Å was observed in both closed and open vesicles when cut perpendicular to the plane of the membrane.

Upon incubation of the membranes with anti-AcChoR $(Fab')_2$ and subsequently with ferritin-labeled goat anti-rabbit IgG, ferritin molecules were found to cover the outer surface of a high proportion of intact vesicles (Fig. 1 *a–d*), whereas some of the vesicles did not show any labeling (Fig. 1*c*). Heavy and uniform labeling with ferritin molecules was found on most of the labeled vesicles (Fig. 1 *a* and *c*). However, occasionally, in larger vesicles the labeling was not regular, some areas being highly labeled and others devoid of labeling (Fig. 1*b*).

In high magnification images of perpendicular sections it was evident that the distance of the ferritin molecules from the surface of the membrane was in the range of 50–150 Å. This distance probably represents the space occupied by the complex of rabbit $(Fab')_2$ fragment and its complementary goat antibody (22). In tangential cuts, dense covering of the vesicle membranes with ferritin molecules was observed (Fig. 1*d*).

The immunoferritin technique using anti-AcChoR antibodies can be an appropriate tool for testing whether antigenic sites of AcChoR molecule are exposed on both sides of the membrane, provided that the specific antibodies and the ferritin-labeled antibodies have an access to the inner face of the membrane. When open profile membranes were examined systematically, we found that labeling was not restricted to one side of the membrane but was usually observed on both sides (Fig. 1 *a* and *b*). Labeling on both sides of the membrane was occasionally detected on closed membrane profiles (Fig. 1*c*). Such profiles may represent unsealed vesicles in which the damage to the vesicle is outside the plane of sectioning.

It should be noted that the use of anti-AcChoR $(Fab')_2$ fragments rather than the whole immunoglobulin fraction markedly reduced the nonspecific staining (data not shown). In control experiments in which the membrane specimen was incubated with unrelated $(Fab')_2$ and subsequently with ferritin-labeled anti-IgG, no labeling of membranes was observed (Fig. 1*e*). Occasionally, ferritin molecules could be observed on cell debris or on amorphous electron-dense material (Fig. 1*e*). In a control experiment in which vesicles were incubated with ferritin-labeled goat anti-rabbit IgG alone, and fixed and processed for the electron microscope without any washing, ferritin was visible mainly in the extravesicular space and did not penetrate significantly into the closed membrane structures (Fig. 1*f*).

the inner side also. In a perpendicular cut of the membrane, the ferritin molecules are visualized at a distance from the membrane not exceeding about 150 Å. (×89,000.) (*b*) Ferritin molecules stain both sides of an open membrane fragment (arrowheads). In large closed membrane profiles, ferritin labeling is evident on the outer side of some parts of the vesicle membrane, whereas other parts are devoid of labeling. In a tangential cut of the membrane, clusters of ferritin molecules can be seen (asterisks). (× 80,000.) (*c*) A rounded membrane profile (A) labeled on both sides of the membrane (arrowheads). No apparent opening is visible at the section plane. In contrast, two other membrane profiles (B and C) are completely devoid of ferritin labeling. (×80,000.) (*d*) Membrane vesicle cut tangentially and heavily labeled with ferritin particles (asterisk). Another vesicle is labeled on the outer surface (arrowhead). (×79,000.) (*e*) Control specimen incubated with nonrelevant rabbit $(Fab')_2$ fragments, and subsequently with ferritin-conjugated goat anti-rabbit IgG. The preparation consists mainly of rounded closed membrane vesicles (V) as well as a few open profiles (wide arrow). Only a few ferritin molecules can be detected on some cell debris (arrowhead). Membrane vesicles are essentially free of ferritin labeling. (Section stained as for *a–d*; ×81,000.) (*f*) Specimen treated with excess of goat anti-rabbit IgG alone without washing prior to fixation. This experiment was performed to test the penetrativeness of the tracer into the vesicles. Ferritin molecules occupy the extra-vesicular space, but the lumen of most vesicles (L) is essentially devoid of the tracer. (Unstained section; ×79,000.)

## DISCUSSION

In this study we have used specific antibodies to AcChoR to localize the receptor molecule in membranes of *Torpedo californica* electroplax, using high-resolution immunoferritin staining. The application of specific anti-AcChoR $(Fab')_2$ fragments in this study, rather than the whole immunoglobulin fraction, reduced markedly the nonspecific staining. The advantages of using $(Fab')_2$ fragments for immunoferritin localization in other systems were reported earlier (23–25).

The data presented in this study suggest that AcChoR is a transmembrane protein, exposing its antigenic sites at both the extracellular and the cytoplasmic faces of the electroplax membrane. This notion was earlier put forward by Changeux *et al.* (3) on the basis of two lines of evidence: (*i*) AcChoR was shown to be involved in the process of ion translocation across the membrane (6, 26); (*ii*) freeze-fracture experiments demonstrated the presence of organized lattices of intramembranal particles tightly bound to the cytoplasmic (A) face of the postsynaptic membrane of *Torpedo* electroplax (11). In another study (12) it was demonstrated that lattice structures of intramembrane particles were mainly associated with the outer leaflet face of this membrane (B face). These particles were of the size and distribution to be expected for the receptor molecule (13, 15). However, no conclusive evidence has previously been available to demonstrate that the receptor is a transmembrane protein, and alternative models could also be drawn from the various ultrastructural studies (3, 9, 11–14). The immunoferritin technique utilizing anti-AcChoR $(Fab')_2$ fragments appeared to be an appropriate tool for approaching this problem in a direct manner. By using the ferritin-stained receptor-rich membrane preparation, three representative types of membrane profiles could be distinguished: (*i*) unlabeled profiles; (*ii*) closed profiles labeled only on the outer side of the vesicle membrane; and (*iii*) open profiles labeled on both sides of the membrane. Our conclusion regarding the bilateral expression of antigenic sites of the receptor is based mainly on the distribution of label in the latter type of unsealed membrane fragments, which readily permitted interaction of the immunochemical reagents with both sides of the membrane. We have not attempted, in this study, to prepare open membrane fragments deliberately by employing detergents, because such treatments could alter the distribution and orientation of the receptor molecule in the membrane. However, one cannot completely rule out the possibility that the sonication step which is essential for preparing the purified membranes, might induce some changes in the organization of the receptor molecules within the membranes.

Concerning the vesicles that were stained only in their outer face, we have demonstrated that the ferritin-conjugated antibodies could not penetrate the lumen of most of the vesicles. Therefore the apparent polarity of the membrane staining probably stems from failure of the immunochemical reagents to penetrate, rather than from the lack of AcChoR antigenic sites on the unstained side of the membrane.

Because we have demonstrated bilateral expression of antigenic sites of AcChoR, AcChoR-containing membranes should have been stained at least on their exposed face. Therefore, it is most likely that those profiles lacking any staining represent membrane fragments devoid of AcChoR. Indeed, ultrastructural analysis of negatively stained receptor-rich membrane fraction of *Torpedo* electroplax revealed that some of the membrane fragments were devoid of particles interpreted to represent AcChoR molecules (9, 11, 13, 14). The presence or exclusion of AcChoR at different areas of the membrane in the electrogenic cell (e.g., innervated and noninnervated sides) results in the formation of either receptor-rich vesicles or receptor-free vesicles. However, in large vesicles differential regional distribution of receptor could be detected.

Our high-resolution immunoferritin localization of AcChoR has allowed the direct study of the mode of insertion of the AcChoR molecule in the excitable membrane. Autoradiographic studies using radiolabeled $\alpha$-bungarotoxin were utilized successfully for both qualitative and quantitative studies of the distribution and density of AcChoR in the post-synaptic membrane (10). However, the elucidation of the molecular organization of AcChoR within the membrane is beyond the resolution of that technique. Our observations implying a bilateral exposure of AcChoR may shed light on earlier ultrastructural studies that used negatively stained or freeze-fractured preparations (9, 11–14). If indeed AcChoR is a transmembranal protein, it is not surprising that a similar pattern of receptor-like particles was observed both on the membrane surface (by negative staining or deep etching) and on the inner membrane face by freeze fracture. Thus, the various methods used for receptor localization probably reveal the pattern of receptor organization at different levels across the membrane.

Antibodies against *Torpedo* AcChoR crossreact with AcChoR of other species (27–29). Therefore, the antibodies can be used as a tool for the fine localization of AcChoR in other species as well. Moreover, immunoferritin localization using antibodies against the intact AcChoR molecule or its subunits may enable us to follow receptor mobility and degradation in the intact cell.

Our data demonstrate that antigenic sites of the immunogen against which the antibodies were raised are located on both sides of the membrane in isolated vesicles. The anti-AcChoR $(Fab')_2$ fragments used in the present study were derived from anti-AcChoR antibodies prepared against a purified AcChoR preparation that is composed of several polypeptides believed to be constituents of the receptor molecule (2–4, 16). However, the possibility cannot be completely excluded that an immunogenic impurity derived from the inner site of the membrane and not intrinsically associated with the receptor molecule could still exist and explain the results.

Antisera to the entire AcChoR molecule probably contain antibody populations directed against different antigenic determinants in the molecule and thus can also recognize regions of the receptor other than the neurotransmitter binding site. The use of antibodies against chemically defined antigenic determinants representing different parts of the molecule [e.g., isolated subunits (30–32)] and antibodies against $\alpha$-neurotoxins will facilitate a more detailed analysis of the insertion and orientation of the AcChoR molecule in the membrane. Such studies may enable us to examine which parts of the molecule are exposed and which are hidden in the bilayer of the membrane in normal tissue or in diseases associated with deranged receptor function.

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