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Topographic studies of *Torpedo* acetylcholine receptor subunits as a transmembrane complex

(sealed vesicles/tryptic hydrolysis/subunit exposure)

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ABSTRACT **The exposure of the four subunits of the acetylcholine receptor from *Torpedo californica* on both the extracellular and cytoplasmic faces of the postsynaptic membranes of the electroplaque cells has been investigated. Sealed membrane vesicles containing no protein components other than the receptor were isolated and were shown to have 95% of their synaptic surfaces facing the medium. The susceptibility of the four receptor subunits in these preparations to hydrolysis by trypsin both from the external and from the internal medium was used to investigate the exposure of the subunits on the synaptic and cytoplasmic surfaces of the membrane. It was shown by sodium dodecyl sulfate gel electrophoresis of the tryptic products that all four subunits are exposed on the extracellular surface to a similar degree. All four subunits are also exposed on the internal surface of the membrane, but the apparent degree of exposure varies with the subunit size, the larger subunits being more exposed. The results are discussed in terms of a possible topographic model of the receptor as a transmembrane protein complex.**

Excitable membrane vesicles highly enriched in the acetylcholine receptor (AcChoR) have been purified from the electric organs of several species of electric fish. These membrane preparations possess the properties of nicotinic postsynaptic membranes: they bind α-neurotoxins (1, 2) and cholinergic ligands (3, 4) and they possess distinct binding sites for local anesthetics (4–7) and the alkaloid histrionicotoxin (8, 9). Binding of cholinergic agonists results in the flux of inorganic cations through the vesicular membrane (10–13), and the demonstration of such flux through membrane preparations containing only the AcChoR protein (14, 15) suggests that the AcChoR functions as a cation-translocating protein complex.

Detergent-extracted, chromatographically purified AcChoR from *Torpedo californica* sediments both as a 9S monomer and as a 13.7S dimer (16). Except for one account (17), both species have been found to consist of four subunits of 40,000, 50,000, 60,000, and 65,000 daltons, with binding sites for α-neurotoxins, agonists, and some antagonists located on the 40,000-dalton subunit (18–30). These four subunits were found to possess a high incidence of amino acid sequence homology (31) and to exist in both membrane-bound and detergent-solubilized receptor as a pentameric complex with a stoichiometry of 2:1:1:1 (31, 32). This stoichiometry dictates a size of 255,000 daltons for the AcChoR monomer, in agreement with the experimentally determined values of 250,000–270,000 daltons (33–35). In light of this molecular definition of the AcChoR and of its defined function alluded to above, it is of interest to determine the topography of its subunits in the membrane and eventually to assign a specific function to each polypeptide.

By use of antibodies to purified AcChoR, it has been found at the electron microscopic level by both thin-sectioning (36) and examination of replicas of intact and sheared membrane vesicles (37) that the receptor is a transmembrane protein, being exposed on both the cytoplasmic and the extracellular surfaces of electroplax membrane vesicles. Which of the four AcChoR subunits are exposed on either of the membrane faces could not be determined by these methods due to the failure of antibodies to individual subunits, isolated from preparative $NaDodSO_4$ gels, to crossreact with membrane-bound receptor.

By use of lactoperoxidase-catalyzed iodination of the AcChoR-enriched, outside-out, sealed membrane vesicles, the 40,000-, 50,000-, and 60,000-dalton subunits were labeled and, thus, exposed on the external synaptic surface of the membrane; the 65,000-dalton subunit was labeled very little under these conditions (ref. 38 and unpublished observations). Both the 40,000- and the 65,000-dalton subunits were labeled with a photolabile derivative of α-bungarotoxin (α-BuTx), indicating their exposure on the exterior membrane surface (39).

In the studies described here, we present an investigation of the topography of the AcChoR molecular complex in the postsynaptic membrane. By using tryptic degradation of the extracellular and cytoplasmic membrane surfaces separately, we have determined that all four of the AcChoR subunits span the membrane bilayer and are exposed on both faces of the postsynaptic membrane.

MATERIALS AND METHODS

Membrane fragments enriched in AcChoR were prepared from *T. californica* electric organs by the method of Elliott *et al.* (25). For further enrichment in AcChoR, the membranes were extracted with base as described (15, 25, 40, 41) and resuspended in 10 mM Tris·HCl (pH 7.4), giving a preparation containing approximately 4 nmol of α-BuTx sites per mg of protein.

The concentration of α-BuTx sites was determined according to Schmidt and Raftery (42) with DEAE-cellulose filter discs and ^{125}I-labeled α-BuTx (43). Protein concentration was determined by the method of Lowry *et al.* (44), with bovine serum albumin as the standard. $NaDodSO_4$/polyacrylamide gel electrophoresis was done according to Laemmli (45) with a 10% (wt/vol) acrylamide/0.27% bisacrylamide separating gel and a 3% (wt/vol) acrylamide/0.08% bisacrylamide stacking gel. After staining with Coomassie brilliant blue, gels were scanned with a Gilford linear transport unit at 550 nm.

For opening and resealing of membrane vesicles, base-treated membrane fragments (1 mg/ml) were subjected to two cycles of freezing and thawing by the method of Moore and Raftery (46) with modification as follows. Membrane fragments (40 μl) in a small Pyrex tube were frozen in liquid nitrogen for 5 min, then thawed at 4°C for 1 min. The second cycle was performed immediately after the first thawing was complete. The sided-

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Abbreviations: AcChoR, acetylcholine receptor; α-BuTx, α-bungarotoxin.

ness of the vesicles was determined with an α-BuTx sites assay as described by Hartig and Raftery (47) for preparations of intact vesicles, except that the buffer used here was 10 mM Tris·HCl (pH 7.4).

Tryptic Degradation of Exterior Surface of Membrane Vesicles. After two cycles of freezing and thawing, membrane vesicles (1 mg/ml) were treated with trypsin (100 nM) for 2 hr at 37°C. Soybean trypsin inhibitor was then added to a final concentration of 50 μM and the sample was heated in gel sample buffer containing $NaDodSO_4$ and 2-mercaptoethanol for electrophoresis. A control sample, in which both the trypsin and the inhibitor were added before the freezing and thawing cycles, was included in each experiment. The time course of the hydrolysis was measured by incubating the samples at 37°C for 1 min to 2 hr; at the appropriate time, inhibitor and sample buffer were added and the sample was heated (100°C for 3 min) and frozen at −20°C until electrophoresis was performed.

Tryptic Degradation of Interior Surface of Membrane Vesicles. Membrane vesicles (1 mg/ml) were added to trypsin (100 nM) at 4°C and immediately frozen in liquid nitrogen for 5 min. After the samples were thawed at 4°C for ≤1 min, the freezing was repeated. Soybean trypsin inhibitor was added (50 μM) immediately upon completion of the second thawing and the sample was incubated at 37°C for 2 hr. As a control each time, a sample was treated as above with trypsin, frozen and thawed twice, treated with inhibitor, and then immediately mixed with gel sample buffer, heated, and frozen until electrophoresis was begun. The time course of the tryptic degradation of the interior surface was measured by quenching with gel sample buffer at the appropriate time, as described above for the degradation of the exterior surface.

RESULTS

After two cycles of freezing and thawing, the membrane vesicle preparations were found to be oriented such that 95% ± 5% (SEM) of their α-BuTx binding sites were exposed to the external solution; i.e. only ≈5% of these vesicles had the extracellular (synaptic) surface of the postsynaptic membrane oriented inward. Neither the number nor the orientation of α-BuTx binding sites changed during 2 hr at 37°C.

Preliminary experiments (data not shown) of degradation of membrane fragments with various concentrations of trypsin, quenching with various concentrations of trypsin inhibitor, and incubation at various temperatures led to the final experimental conditions described above. It was found that incubation with concentrations greater than 100 nM trypsin resulted in an increased rate of degradation to the point where 1 min at 4°C was sufficient for significant degradation to occur. A slower rate was desired to permit thawing of the samples to which trypsin had been added to the interior before addition of inhibitor to the outside such that no significant degradation of the outer surface of the membrane occurred. Likewise, thawing at 4°C was used to avoid the noticeable degradation that occurred at room temperature or at 37°C in 1 min. The large excess of inhibitor (50 μM inhibitor for 100 nM trypsin) used to quench the hydrolytic reaction was necessary to obtain immediate quenching; with even 10% less inhibitor, a significant amount of hydrolysis occurred after addition of inhibitor. Two cycles of freeze-thawing were found to be sufficient to load the trypsin into the vesicles; one cycle resulted in less complete degradation of AcChoR subunits, but no further improvement was observed with three cycles.

When vesicles were treated with trypsin on the outside or inside surfaces and the membrane proteins were electrophoresed on $NaDodSO_4$ gels, the results shown in Fig. 1 were obtained. Neither the freeze-thawing process nor the incubation with trypsin plus inhibitor for 2 hr at 37°C had any effect on the gel pattern (compare tracks A and E). When trypsin was added after the freeze-thawing so that only the extracellular surface of the vesicles was exposed to the enzyme, all four AcChoR subunits were degraded, as can be seen from the decrease in their staining intensity in track B. At the same time new bands appeared on the gel: a broad band at ≈45,000 daltons and sharper ones at ≈34,000, ≈30,000, and ≈27,000 daltons (track B). In a parallel experiment, trypsin was loaded into the vesicles by freeze-thawing, and degradation of the exterior surface was prevented by the addition of trypsin inhibitor to the thawed solution of resealed vesicles; in this way, only the cytoplasmic face of the postsynaptic membrane was exposed to trypsin. The results of this experiment are shown in tracks C and D of Fig. 1. In the control sample, shown in track C, gel sample buffer was added to the sample immediately after the thawing and addition of inhibitor. Comparison of this control with track E (no trypsin added) shows that little degradation occurred during the thawing period, when trypsin but not the inhibitor was present both inside and outside the vesicles. Only a small band at ≈34,000 daltons indicates that any degradation at all occurred during this period, and the staining intensity of none of the subunit bands decreased measureably. In track D, the results of a 2-hr incubation at 37°C with trypsin sealed inside the vesicles and trypsin–inhibitor complex present outside are shown. The staining intensities of the 65,000-, 60,000-, and 50,000-dalton bands decreased considerably, while that of the 40,000-dalton band decreased a little. New bands also appeared on the gel: a broad band at ≈48,000 daltons and sharp bands at ≈38,000, ≈37,000, ≈34,000, ≈33,000, and ≈31,000 daltons.

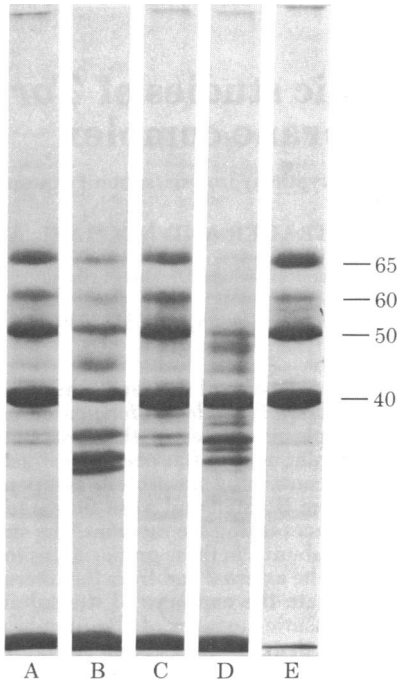

FIG. 1. $NaDodSO_4$ gel electrophoresis of: (track A) membrane fragments treated with trypsin and inhibitor, subjected to two cycles of freezing and thawing, and incubated at 37°C for 2 hr; (track B) membrane fragments treated with trypsin after freeze-thawing, then incubated at 37°C for 2 hr (outside tryptic hydrolysis); (track C) membrane fragments treated with trypsin, then frozen and thawed, treated with inhibitor immediately after thawing, then heated in gel sample buffer; (track D) same as track C, but incubated at 37°C for 2 hr instead of being heated in gel sample buffer (inside tryptic hydrolysis); and (track E) untreated membrane fragments. The four AcChoR subunits are marked in kilodaltons.

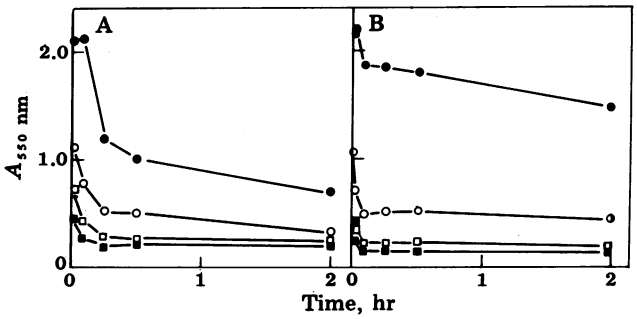

FIG. 2. Time course of tryptic degradation from the outside (*A*) and from the inside (*B*) of the membrane vesicles. Peak heights from scans of Coomassie brilliant blue-stained gels are given for the 40,000- (●), 50,000- (○), 60,000- (■), and 65,000- (□) dalton subunits.

The time course of a typical experiment is shown in Fig. 2. Tryptic hydrolysis of the protein on the extracellular surface of the vesicles (Fig. 2*A*) proceeded with approximately the same time course for all four subunits, with the most rapid proteolysis occurring during the first 15 min. After this time, degradation of the 60,000- and 65,000-dalton subunits had almost reached its limit, whereas the 40,000- and 50,000-dalton subunits continued to disappear more slowly. On the cytoplasmic electroplaque membrane surface, degradation of the protein proceeded at a rapid initial rate as well (Fig. 2*B*), with degradation of the 50,000-, 60,000-, and 65,000-dalton subunits being almost complete after 5 min. The 40,000-dalton subunit showed little degradation during this period, however, and continued to disappear at a slow rate during the 2-hr incubation period. Although the quantitative data varied from one experiment to the next, with maximal hydrolysis of the 60,000- and 65,000-dalton subunits on either face of the membrane being reached 5–30 min after the incubation was begun, the qualitative results remained consistent; a fast initial hydrolysis was observed for all four subunits, followed by the slower disappearance of the 40,000- and 50,000-dalton subunits, which was especially noticeable for the interior surface of the membrane.

The time courses of tryptic effects on both sides of the membrane were determined on three separate preparations and averaged (each normalized to its control sample) in terms of the percentage of each subunit remaining at a given time (Fig. 3). On the extracellular side of the membrane, all four subunits disappeared at roughly the same rate, with all reaching approximately the same residual level (27–33% of original) after 2 hr. On the cytoplasmic face of the membrane, however, there were marked differences in the rates and degrees of degradation of the four subunits. The 65,000-dalton subunit was the most thoroughly degraded; only 12% of the original Coomassie brilliant blue-staining material remained after 2 hr. Hydrolysis of the 60,000-dalton subunit was almost as complete, with 20% remaining after 2 hr. The 50,000-dalton subunit disappeared more slowly, with 48% of the original remaining after 2 hr; the 40,000-dalton subunit was removed even more slowly, with 70% remaining after 2 hr.

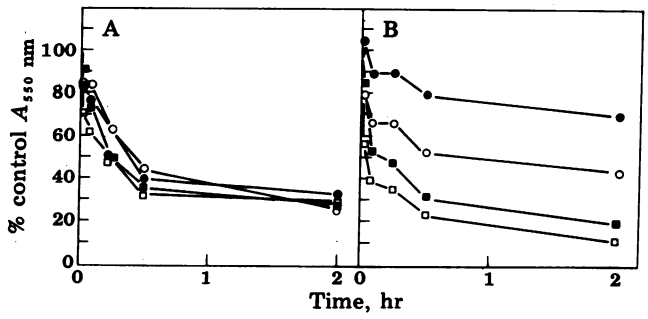

FIG. 3. Time course of tryptic degradation from the outside (*A*) and from the inside (*B*) of the membrane vesicles. Peak heights from scans of Coomassie brilliant blue-stained gels are given as the average of three experiments, each normalized to its own controls. The 40,000- (●), 50,000- (○), 60,000- (■), and 65,000- (□) dalton subunits are shown.

Examination of membrane vesicles by negative-stain electron microscopy showed no effect on the characteristic rosette structure of the AcChoR molecule (18, 48–50) either by the freeze-thawing process or by tryptic hydrolysis from either membrane face.

DISCUSSION

The experimental evidence presented here indicates that all four of the AcChoR subunits span the postsynaptic membrane, being exposed on both the extracellular and the cytoplasmic faces of the membrane bilayer. The freeze-thaw technique for introduction of agents within sealed vesicles results, as we show here, in preparations that are oriented with 95% of the α-BuTx-binding sites outside; i.e., 95% extracellular (synaptic) side facing out. The tryptic hydrolysis of subunits observed from inside the vesicles did not result from residual inside-out vesicles because much more than 5% of the Coomassie brilliant blue-staining material for each subunit disappeared from the gel in all cases, whereas only 5% of the vesicles were inside-out. Unsealed vesicles, whether oriented inside-out or outside-out, would not be detected by the method used. α-BuTx would have access to either side of such vesicles, but these membranes would not be degraded under the experimental conditions used because trypsin inhibitor added to the solution would also have access to both sides of these open vesicles. Thus, only the sealed membrane vesicles, 95% of which were oriented with their extracellular sides facing outward, constituted the population of vesicles studied in our experiments.

Under the conditions of the experiments, all four subunits were hydrolyzed to some extent from both the extracellular and the cytoplasmic sides of the vesicles, indicating their exposure on both faces of the membrane and implying that all four subunits span the membrane bilayer and traverse the hydrophobic core of the membrane. In experiments designed to determine which of the AcChoR subunits penetrate into the hydrophobic membrane bilayer, Sator *et al.* (51) used the lipophilic photolabel [^{3}H]pyrenesulfonylazide to label membrane-bound AcChoR and determined that the 50,000- and 60,000-dalton subunits were exposed to the hydrophobic membrane core. In similar experiments using 5-[^{125}I]iodonaphthyl-1-azide as the lipophilic label, Tarrab-Hazdai *et al.* (52) found that only the 40,000-dalton subunit was exposed to the hydrocarbon interior of the membrane bilayer.

Evidence that different domains of the subunits are exposed on the two faces of the membrane was obtained from inspection of the gels of the tryptic degradation patterns (Fig. 1). The Coomassie brilliant blue-staining patterns of the samples treated on the outside and the inside with trypsin were quite different (tracks B and D). Although at least one degradation product appeared common to both, at least on the basis of size (≈34,000 daltons), many distinct products were obtained in each case. This diversity in the new peptides produced upon tryptic hydrolysis of the AcChoR from the extracellular face in contrast to the cytoplasmic face of the membrane demonstrates clearly that the distribution of the subunits in the postsynaptic membrane is asymmetric, with different domains being exposed on each side of the membrane.

From a comparison of the time course of the degradative processes on the two sides of the membrane, qualitative esti-

mates of the relative degrees of exposure of trypsin-sensitive residues for each subunit may be made. On the extracellular membrane face, all four subunits disappeared at roughly the same rate, indicating that, with respect to hydrolysis by trypsin, they are all exposed to approximately the same degree. On the cytoplasmic face, however, after the initial rapid hydrolysis the 65,000-dalton subunit was degraded the most readily, followed closely by the 60,000-dalton subunit, with the 50,000-dalton protein disappearing more slowly and the 40,000-dalton subunit much more slowly still. This indicates that, with respect to trypsin-sensitive residues, the 65,000- and 60,000-dalton subunits are the most exposed on the cytoplasmic face of the membrane, followed by the 50,000-dalton subunit, with the 40,000-dalton subunit exposed very little. More evidence is needed with probes for other amino acids before it will be possible to state whether this differential degree of exposure on the cytoplasmic face is in fact the case for the four subunits or is merely a reflection of different sensitivities to trypsin of the exposed regions.

Although we cannot completely rule out the possibility that morphological changes were responsible for the exposure of some of the subunits to tryptic hydrolysis, this does not seem likely. The AcChoR rosettes appeared at the electron microscopic level to be unaffected by treatment with trypsin. Moreover, if tryptic degradation led to the opening of holes or tears in the membrane to allow hydrolysis of subunits that were not initially exposed on the membrane surface, then the trypsin inhibitor on the outside would reach the trypsin inside the vesicle and quench the reaction.

In summary, we find that all four AcChoR subunits span the postsynaptic membrane based on the following lines of evidence. The membrane preparations used in these studies consist of sealed vesicles, 95% of which are oriented with the extracellular (α-BuTx binding) side facing outward. Thus, the preparations are well characterized, with the exterior side of the vesicles corresponding to the extracellular face of the postsynaptic membrane and the interior side corresponding to the cytoplasmic membrane face. Tryptic degradation of the proteins exposed on either of the two sides of the vesicles produces a decrease in Coomassie brilliant blue-staining intensity of all four subunits from both faces of the membrane with concomitant appearance of several degradation products; the degradation products appearing on the gels are strikingly different for tryptic hydrolysis from the two membrane faces. Moreover, the rate of disappearance of the AcChoR subunits is different for tryptic degradation from the two sides of the membrane, with all four polypeptide chains disappearing at approximately the same rate from the vesicle exterior and at vastly differing rates from the interior surface of the vesicular membranes. Thus, it is clear that all four AcChoR subunits span the postsynaptic membrane, with different domains of each subunit being exposed on the two membrane faces.

At the ultrastructural level, high-resolution electron micrographs of the AcChoR rosettes show from four to six similar "subunits" arranged about an electron-dense core (18, 48–50). This is consistent with recent reports that the receptor molecule consists of four homologous subunits present in the ratio 2:1:1:1 (31, 32), for a total of five similar subunits, all exposed on the extracellular surface of the membrane. The four subunits show a considerable amount of sequence homology at their amino termini (31); however, it is not known whether the homology continues through the carboxyl termini of all four polypeptides. The similar rates of tryptic degradation of the four subunits on the exterior of the membrane, when compared to the vastly differing rates of degradation observed for the four on the vesicular interior, suggest that the four subunits are, by this criterion, equally exposed on the extracellular face of the membrane; by the same criterion, the longer chains are more exposed and the shortest (40,000-dalton) least exposed on the cytoplasmic membrane face. This evidence is consistent with a model of the AcChoR as a pentameric structure of two identical and three pseudoidentical polypeptide chains on the exterior face of the membrane and continuing to an unknown extent within the bilayer. This complex extends through the membrane, with the additional carboxy-terminal portions of the longer chains exposed at least partially on the cytoplasmic face and the carboxyl terminus of the shortest chain barely exposed on the interior surface of the membrane.

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