Identification of Exposed and Buried Determinants of the Membrane-Bound Acetylcholine Receptor from *Torpedo californica*†

Stanley C. Froehner

ABSTRACT: The ability of five rabbit anti-acetylcholine receptor antisera to recognize the membrane-bound receptor from *Torpedo californica* has been investigated. Two antisera, raised against affinity-purified native receptor, react extensively with purified receptor-rich membrane vesicles. Since the membrane vesicles are impermeable to macromolecules and are oriented right side out, these two antisera recognize predominantly extracellular determinants. Two antisera against sodium dodecyl sulfate denatured receptor and one against purified δ subunit react poorly with the membrane-bound receptor. Only 10–20% of the determinants recognized by these antisera are accessible to antibodies when the receptor is membrane bound. Many of the latent sites can be exposed by permeabilizing the vesicles with saponin, by alkaline extraction of the membranes to remove peripheral proteins, or by a combination of these two treatments. These treatments neither solubilize the receptors nor interfere with their ability to undergo agonist-induced affinity changes. Subunit analysis of the sites on the membrane-bound receptor that are accessible to antibodies indicates that the α, β, and δ chains possess extracellular determinants. Buried sites are present on all four of the subunits. Saponin permeabilization makes latent sites accessible on α and δ while alkaline extraction uncovers determinants on α, γ, and δ. Treatment of membranes by both procedures reveals sites on β, γ, and δ that are not uncovered by either treatment alone. This study, in conjunction with results from other laboratories demonstrating that the γ chain is extracellularly exposed, suggests that all four subunits are transmembrane proteins.

The nicotinic acetylcholine (ACh)[1] receptor binds cholinergic ligands and mediates a transient increase in the permeability of the membrane to Na^+ and K^+. In recent years, the ACh receptor has been studied extensively in both its membrane-bound and solubilized, purified forms [for a recent review, see Karlin (1980)]. The purified receptor from *Torpedo* electric tissue is a large integral membrane protein complex with a molecular weight of approximately 250 000 (Reynolds & Karlin, 1978), and a Stokes radius of ~72 Å (Raftery et al., 1972; Reynolds & Karlin, 1978). This complex appears to possess the components necessary for both the transmitter binding event and the membrane permeability changes as demonstrated by agonist-induced Na^+ flux assays on highly purified membrane preparations (Neubig et al., 1979; Elliot et al., 1980) and on receptors reconstituted into lipid vesicles (Epstein & Racker, 1978; Lindstrom et al., 1980a). Gel electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$) of the purified receptors reveals that the complex is composed of polypeptides of apparent molecular weights of 40 000 (α), 50 000 (β), 60 000 (γ), and 65 000 (δ) (Weill et al., 1974; Hucho et al., 1976; Chang & Bock, 1977; Froehner & Rafto, 1979; Lindstrom et al., 1979a; Vandlen et al., 1979) that are present in a molar ratio of 2:1:1:1, respectively (Reynolds & Karlin, 1978; Lindstrom et al., 1979a). The absence of one or more of the larger polypeptides from some preparations (Sobel et al., 1977) is likely due to proteolysis. Inhibition of proteolysis during isolation of the receptor is important in order to achieve reproducible subunit patterns (Froehner & Rafto, 1979; Lindstrom et al., 1980b).

Subunit function has been a major focus of several research groups. There is universal agreement that the α subunit possesses the cholinergic binding site. It can be affinity labeled with both 4-*N*-maleimidobenzyl[^{3}H]trimethylammonium iodide (Weill et al., 1974) and [^{3}H]bromoacetylcholine (Damle et al., 1978; Moore & Raftery, 1979) after reduction and also with [^{3}H]-*p*-(trimethylammonium)benzenediazonium fluoroborate (Weiland et al., 1979). The functions of the other polypeptides remain to be determined. Since the ACh receptor modulates ion permeability by a channel mechanism (Karlin, 1973), one or more of the subunits must span the membrane. Recent studies of the proteolytic sensitivity of the subunits of the receptor in its membrane-bound form have addressed this point (Wennogle & Changeux, 1980; Strader & Raftery, 1980).

In this report, we present the results of investigations of the reactivity of anti-ACh receptor (anti-AChR) antisera with the receptor in its membrane-bound form. These studies were conducted for two reasons. First, it seemed feasible to study receptor topology in the membrane by identifying and characterizing two general types of antigenic determinants: those that are accessible to antibodies when the receptor is membrane bound and those that are not. The basis for inaccessibility of buried sites could then be investigated by treating the membranes in various ways to expose them. Determination of the subunits which possess buried sites would provide additional information about the topology of the receptor components.

Second, the ability of antibodies to bind to the receptor in situ is of importance in the human neuromuscular disease myasthenia gravis and in its experimentally induced counterpart experimental autoimmune myasthenia gravis (EAMG) [for a recent review, see Lindstrom (1979)]. Although much is known about the reactivity of anti-AChR antibodies with detergent-solubilized receptor, little quantitative data on the

† From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755. *Received February 24, 1981.* This work was supported by a grant from the Muscular Dystrophy Association and by National Institutes of Health Grant NS14871.

[1] Abbreviations used: ACh, acetylcholine; AChR, acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; αBuTx, α-bungarotoxin; anti-nAChR, antiserum against native AChR; anti-dAChR, antiserum against denatured AChR; anti-δ, antiserum against the δ subunit; BSA, bovine serum albumin.

0006-2960/81/0420-4905$01.25/0

binding of these antibodies to the membrane-associated receptors are available. In view of the observation that injection of native receptor into rabbits induces EAMG while denatured receptor does not (Valderrama et al., 1976; Bartfeld & Fuchs, 1977), it seemed important to compare the ability of such antisera to recognize the membrane-bound receptor.

Experimental Procedures

Preparation of Affinity-Purified AChR. AChR was purified from liquid nitrogen frozen *Torpedo californica* electric organ (Pacific Biomarine) as described (Froehner & Rafto, 1979) with some modifications. Extraction of the crude membrane fraction was done in the presence of 10 mM *N*-ethylmaleimide (NEM) with 2% cholate instead of Triton. Also, cholate was used in all buffers instead of Triton but at the same concentrations. After elution from the column, the purified AChR was dialyzed extensively against homogenization buffer containing 1 mM NEM and 0.01% cholate and stored at 4 °C. AChR used for the production of antisera was prepared without NEM exactly as described by Froehner & Rafto (1979).

Preparation of AChR-Rich Membranes. Live *Torpedo californica* rays were obtained from Pacific Biomarine. Membranes were isolated from the electric organ by the procedure described by Sobel et al. (1977) with the following modifications. Homogenization of the tissue was done in a large Waring blender in the cold for four periods of 1 min each. The homogenate was cooled to 9–10 °C between homogenization periods. Both sucrose-gradient purifications were performed in an SW27 rotor at 23 000 rpm for 6–8 h. The sonication steps were omitted.

Treatment of membranes with saponin was performed as described by St. John et al. (1979). Membranes (1.1 mg/mL protein) in 10 mM $NaPO_4$, pH 7.4, 0.15 M NaCl, and 0.02% NaN_3 (PBS) were mixed with $^1/_9$ volume of 1% saponin at 4 °C. After 10 min, the membranes were pelleted by centrifugation at 25000*g* for 20 min. Pellets were resuspended by gentle homogenization with a Dounce homogenizer in PBS and collected by centrifugation. After resuspension in PBS, the saponin-treated membranes were dialyzed against PBS and stored at 4 °C.

Alkaline extraction of normal or saponin-treated membranes was performed as described by Neubig et al. (1979). The treated membranes were resuspended in PBS, dialyzed against PBS, and stored at 4 °C. Protein concentrations were determined according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

Production of Antisera. All antisera were raised in rabbits. For the preparation of antibodies to native AChR, approximately 100 μg of affinity-purified AChR in 2.0 mL of 0.05 M Tris, pH 7.4, 0.05 M NaCl, and 0.25% Triton was emulsified with a equal volume of complete Freund's adjuvant and injected intradermally and subcutaneously in multiple sites along the back. This was repeated 21 days later using incomplete Freund's adjuvant. The animals were bled 5–7 days later. For the preparation of antibodies to the denatured receptor, the protein was denatured by boiling in 1.6% $NaDodSO_4$ for 5 min before emulsification and injected according to the same protocol.

AChR subunits (~100 μg of each) were separated by $NaDodSO_4$ gel electrophoresis (see below). The gel was stained briefly with Coomassie Blue, and the protein bands were excised with a razor blade. After equilibration in PBS, the gel strips were homogenized in a Dounce homogenizer, mixed with about 2 mL of PBS, and emulsified with an equal volume of complete Freund's adjuvant. Animals were injected as described above. Subsequent injections were carried out with incomplete Freund's adjuvant 23, 78, and 125 days later. Animals were bled at various times during this period, but the most active serum was obtained on day 135. This procedure was successful only with the δ subunit.

Anti-αBuTx was prepared according to the procedure of Daniels & Vogel (1978). All sera were heat inactivated at 60 °C for 20 min and stored at −70 °C.

Radioiodination of Proteins. For the preparation of α-[^{125}I]bungarotoxin ([^{125}I]αBuTx), 5 mCi of carrier-free Na^{125}I (Amersham), 2.5 nmol of NaI, 5 nmol of chloramine-T, and 7 nmol of H_2SO_4 in a total volume of 72 μL were incubated for 1 min on ice in a well-ventilated hood. αBuTx (5 nmol) in 70 μL of 0.77 M $NaPO_4$, pH 7.4, was added, and after 2 min at 4 °C, the reaction was stopped by the addition of 10 μL of 1 mM dithiothreitol and 50 μL of 100 mM NaI. Labeled αBuTx was then separated from the reactants on a small Bio-Gel P6 column equilibrated with 3 mM $NaPO_4$, pH 7.4, and 1 mg/mL BSA. The monoiodinated αBuTx was then purified according to Vogel et al. (1972).

AChR was radioiodinated by the glucose oxidase–lactoperoxidase method. Affinity-purified AChR (1 mg) was mixed with 5 mCi of carrier-free Na^{125}I, 150 nmol of NaI, and 100 μL of Enzymobeads (Bio-Rad) in a total volume of 2.9 mL of 0.10 M $NaPO_4$, pH 7.4. The reaction was initiated by the addition of 100 μL of 10% D-glucose and continued for 15 min at room temperature. After the addition of cholate to 1%, the sample was centrifuged briefly in a microfuge to remove the Enzymobeads. For determination of the specific radioactivity of the labeled protein, acid-precipitable radioactivity of an aliquot of the sample was determined by a filter disk method (Hubbard & Cohn, 1975). Recovery of protein was assumed to be 100% at this stage. The remainder of the sample was then purified on a 1.5 × 26 cm Bio-Gel P-100 column equilibrated with PBS containing 0.1% cholate. Radioactive fractions in the excluded volume were pooled and stored at 4 °C. Specific radioactivities were (1.5–7) × 10^5 dpm/pmol.

^{125}I-Labeled protein A was prepared by a modified chloramine-T procedure (Greenwood et al., 1963). Protein A (50 μg) was mixed with 2 mCi of carrier-free Na^{125}I and 13 nmol of chloramine-T in a final volume of 90 μL of 0.10 M $NaPO_4$, pH 7.4. After 2 min at 4 °C, the reaction was terminated with 10 μL of sodium metabisulfite (1 mg/mL), and the sample was applied to a 1 × 8 cm Bio-Gel P-10 column equilibrated with PBS. Radioactive fractions in the excluded volume were pooled and stored at −70 °C. Specific radioactivity was (3–6) × 10^7 dpm/μg.

Assays for [^{125}I]αBuTx Binding to AChR. Binding of [^{125}I]αBuTx to detergent-solubilized AChR was measured as previously described (Froehner & Rafto, 1979). Binding to AChR-rich membranes was measured by incubating membranes with a 5–10-fold excess of [^{125}I]αBuTx in a total volume of 25 μL of PBS containing 1 mg/mL BSA for 45 min at 37 °C. Reaction mixtures were then diluted with 3 mL of PBS–BSA and collected on filters (0.2 μm EGWP Millipore). The filters were washed 3 times with PBS–BSA and then counted in a Beckman 4000 γ counter.

Assays for Antibody Activities. Anti-AChR activity was determined by immunoprecipitation of [^{125}I]AChR. Duplicate samples of [^{125}I]AChR (1.0–2.0 pmol) in 10 μL of 10 mM $NaPO_4$, pH 7.4, 0.15 M NaCl, 0.02% NaN_3, 0.1% Triton X-100, and 1 mg/mL hemoglobin (PBST) were mixed with 10 μL of antiserum diluted in PBST and incubated for 30 min at 37 °C. IgGsorb (10 μL) (fixed *Staphylococcus aureus* bacteria; The Enzyme Center, Boston, MA) was added, and

the incubation was continued for 30 min. Reaction mixtures were then diluted with 1 mL of PBST and centrifuged in an Eppendorff 5413 microfuge for 10 min. The pellets were washed twice by resuspension in 1 mL of PBST with vortexing and centrifugation. Radioactive antigen–antibody complexes bound via protein A to the fixed bacterial cells were measured in γ counter. Anti-αBuTx activity was determined in an identical manner using [^{125}I]αBuTx (0.25 pmol) as the antigen.

Assay for Antibody Binding to AChR-Rich Membranes. Binding of antibodies to membranes was measured by incubating aliquots of antiserum with membranes, removal of bound antibodies by centrifugation, and determination of unbound antibodies by immunoassay. Antiserum (5–20 μL) was incubated with membranes (5–100 μg of protein) in a total volume of 200 μL of PBS at 37 °C with occasional mixing. After 60 min, the membranes were pelleted by centrifugation for 30 min at 4 °C in an Eppendorff microfuge, and the supernatants were carefully removed. Antibody activity in the supernatants was then determined as described above. The same amount of activity (as measured by using [^{125}I]AChR) was used when reacting different antisera with membranes.

Determination of Subunit Specificity of Antisera. The method of Burridge (1976) as modified by Adair et al. (1978) for detecting antigens in $NaDodSO_4$ gels was used. Proteins were separated by $NaDodSO_4$ microslab gel electrophoresis as described by Matsudaira & Burgess (1978). Protein bands were located by reversibly staining the gel with Coomassie Brilliant Blue G as described by Blakesley & Boezi (1977). Lanes (5–7 mm wide) containing the protein bands were cut out, the bottom left corner of each slice was nicked to mark the bottom of the gel, and the strips were then destained in methanol:H_2O:acetic acid (5:5:1) for several hours. After equilibration in buffer A (50 mM Tris, pH 7.4, 50 mM NaCl, and 0.1% NaN_3) for several hours, the strips were transferred to polystyrene tubes (Falcon 2054) containing 3 mL of gelatin solution (1 mg/mL buffer A). Antiserum was added (final dilution of 1:30–1:500), and the capped tubes were incubated in a horizontal position for 24 h at 23 °C with gentle shaking. Care was taken to ensure that the gel strips remained submerged in the buffer throughout the incubation. The strips were then transferred to 50-mL capped tubes containing buffer A and washed for 3 days with gentle shaking. The buffer was changed twice daily. The washed gel strips were then incubated with ^{125}I-labeled protein A (0.75–1.0 μg in 3 mL of gelatin solution) and washed exactly as described above for the antisera. Gel strips were then dried down between dialysis tubing (Bio-Rad) and exposed to Cronex 2DC X-ray film with an intensifying screen at −70 °C.

In some experiments, total cellular protein of the electroplax organ was used as the source of antigen. Approximately 10 g of frozen *Torpedo* electroplax tissue was placed in 10 mL of 0.125 M Tris, pH 6.8, 1 mM EDTA, 1 mM EGTA, Trasylol (10 units/mL), 10 mM NEM, 5 μg/mL pepstatin, and 0.5 mM phenylmethanesulfonyl fluoride and homogenized on ice with a Polytron. $NaDodSO_4$ was added to a final concentration of 2%, and the sample was immediately boiled for 10 min. After centrifugation for 5 min at 30000*g*, glycerol and bromophenol blue were added to final concentrations of 9% and 0.001%, respectively, and the sample was applied to the microslab gel without prior reduction.

Results

Reactivity of Antisera with Detergent-Solubilized AChR. Four rabbits were injected with affinity-purified AChR, either in Triton or after $NaDodSO_4$ denaturation. All four antisera showed high titers of anti-AChR activity when assayed with native [^{125}I]AChR (Figure 1A). The two antisera prepared aganist native AChR (anti-nAChR) showed somewhat higher activity than did the antisera prepared against $NaDodSO_4$-denatured AChR (anti-dAChR). Using values of [^{125}I]AChR precipitated that are linear with the amount of antiserum added, it was determined that anti-nAChR 1 and anti-nAChR 2 precipitate 54 and 23 pmol of AChR/μL of antiserum, respectively. The activities of anti-dAChR 1 and anti-dAChR 2 were 24 and 16 pmol/μL, respectively. The activities of all four antisera were greatly decreased (10–50-fold) when denatured AChR was used as the antigen (Figure 1B). Furthermore, both anti-dAChR sera were more active (about 2-fold) against denatured antigen than were the anti-nAChR sera. Neither native nor denatured AChR was precipitated by rabbit anti-αBuTx (Figure 1). In all subsequent assays, native [^{125}I]AChR and amounts of antiserum that bound 10–20% of the antigen were used.

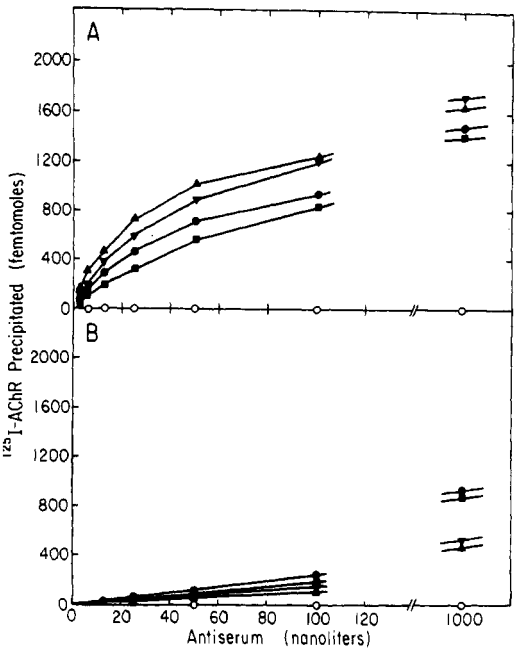

FIGURE 1: Reactivity of antisera with solubilized, purified [^{125}I]AChR. (A) Native [^{125}I]AChR or (B) denatured [^{125}I]AChR (1.8 pmol) was incubated with dilutions of anti-nAChR 1 (▲), anti-nAChR 2 (▼), anti-dAChR 1 (●), anti-dAChR 2 (■), or anti-αBuTx (○) and processed as described under Experimental Procedures.

The production of rabbit antisera of the individual subunits of AChR was not as successful as with the complex. All four subunits were purified and injected individually using several protocols, but only one high-titer antiserum was obtained. The δ polypeptide elicited an activity against native [^{125}I]AChR comparable to that of the anti-dAChR sera (8.5 pmol/μL).

Both rabbits injected with native AChR displayed muscle weakness symptoms characteristic of experimental autoimmune myasthenia gravis (Patrick & Lindstrom, 1973). At the time the serum was taken, the rabbit from which anti-nAChR 1 was obtained was extremely weak and was unable to stand or sit. The symptoms in the second rabbit were less dramatic. In agreement with results from other laboratories (Valderrama et al., 1976; Bartfeld & Fuchs, 1977), neither animal injected with denatured AChR developed symptoms of the disease, even after subsequent injections. Similarly, the rabbit that produced a high titer antiserum to the δ subunit exhibited no symptoms, again in agreement with previous results (Claudio & Raftery, 1977; Lindstrom et al., 1978). Since the effectiveness of anti-AChR antibodies in promoting defective neuromuscular transmission must depend in part on their ability to bind to the receptor in situ, we sought ways

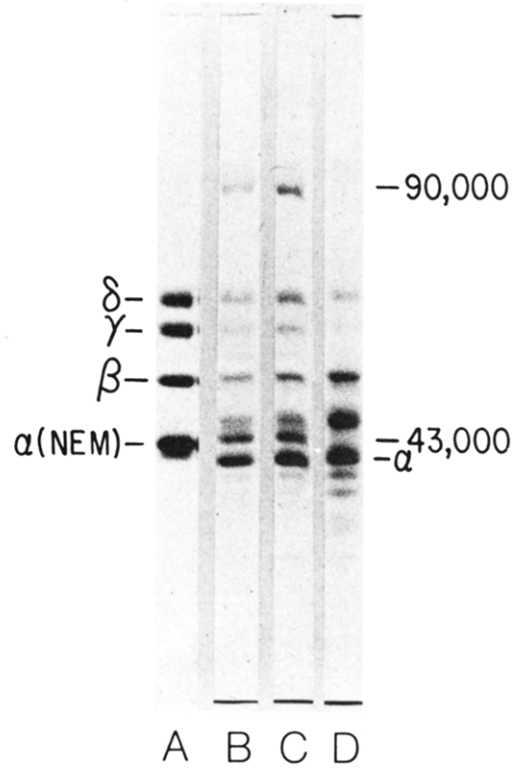

FIGURE 2: $NaDodSO_4$ gel electrophoresis of (A) affinity-purified AChR, (B) AChR-rich membranes, (C) saponin-treated AChR-rich membranes, and (D) alkaline-extracted AChR-rich membranes.

to measure the binding of anti-AChR antibodies to the receptor in its membrane-bound form. Although it would be most appropriate to use postsynaptic membranes from mammalian muscle for these studies, the small amounts present in this tissue preclude their isolation and use in studies of this type. Alternatively, we purified AChR-rich membranes from *Torpedo californica* electric organ and investigated the binding of the antibodies to the homologous antigen. In view of the biochemical similarities between *Torpedo* and mammalian muscle receptors (Froehner et al., 1977; Nathanson & Hall, 1979), this approach seems appropriate.

Characterization of Receptor-Rich Membranes. Receptor-rich membranes prepared by the method of Sobel et al. (1977) are in vesicular form with almost all vesicles oriented in right-side-out conformation. Since about 90% of these vesicles are impermeable to macromolecules, the predominant antigenic determinants available for antibody binding are those normally exposed to the exterior of the cell. Treatment with saponin at low concentrations permeabilizes the vesicles without solubilizing the receptor, thus promoting antibody access to intracellular determinants (St. John et al., 1979). These are essential properties for the study of subunit topology, and it was for these reasons that we used membranes prepared in this manner.

Gel electrophoresis in $NaDodSO_4$ of the AChR-rich membranes is shown in Figure 2. In contrast to some reports (Sobel et al., 1977) but in reasonable agreement with others (Neubig et al., 1979; Wennogle & Changeux, 1980; Froehner et al., 1981), our preparations contain all four subunits of the receptor, although the amount of the γ chain is considerably less than that found in affinity-purified AChR (compare Figure 2A,B). This polypeptide is highly sensitive to proteolysis during isolation, especially if NEM is not present (Froehner & Rafto, 1979). In one preparation, we included NEM in the homogenization buffers but found that the purified membranes possessed substantial amounts of contaminating proteins. The α chain of affinity-purified AChR migrates more slowly than its counterpart in membranes because of its reactivity with NEM (Froehner & Rafto, 1979). In addition to the four receptor polypeptides, the membranes contain two polypeptides which migrate between α and β. The larger of these is probably a degradation product of one of the receptor subunits since it is reactive with anti-AChR (Froehner et al., 1981). Even though some proteolysis of these membranes occurs during isolation, this is unlikely to affect their ability to bind antibodies. Lindstrom et al. (1980b) have shown that membrane-bound receptors that have been almost completely proteolyzed with papain still retain their normal structural and functional properties.

In agreement with other reports (Neubig et al., 1979; Elliot et al., 1980), the polypeptide of approximately 43 000 molecular weight can be removed by extraction of the membranes at pH 11 (Figure 2D) or with lithium diiodosalicylate (Elliot et al., 1980; S. C. Froehner, unpublished experiments). Alkaline extraction also removes two polypeptides of 50 000–55 000 daltons, and greatly diminishes one of about 90 000. Other polypeptides are also present in small amounts, some of which are removed by alkaline extraction. Saponin treatment has little effect on the polypeptide composition of the membranes (Figure 2C). In some preparations, the amount of the 43 000-dalton polypeptide was somewhat reduced by saponin treatment. In most, it was fully retained. The reason for this variability is unknown. Membranes subjected to both alkaline extraction and saponin treatment possess the same composition of polypeptides as those receiving only alkaline extraction (not shown).

Several lines of evidence indicate that these treatments do not grossly alter the functional aspects of the receptor. The functional characteristics of alkaline-extracted AChR-rich membranes are very similar to those of untreated membranes (Neubig et al., 1979; Moore et al., 1979; Elliot et al., 1980). They exhibit agonist-induced Na^+ flux, αBuTx binding, local anesthetic binding, and desensitization properties comparable to those of normal membranes. The saponin treatment used to permeabilize the vesicles precludes investigations of agonist-induced cation flux. However, the slower transition from low- to high-affinity agonist binding, generally thought to represent desensitization (Weiland et al., 1977; Quast et al., 1978), can be studied in permeabilized membranes. As shown in Figure 3, normal membranes as well as those subjected to all three treatments exhibit an increase in affinity for agonists when incubated with carbamoylcholine. If carbamoylcholine (1 μM) and [^{125}I]αBuTx are added simultaneously, the rate of toxin binding is reduced only slightly. Membranes preincubated for 15 min with 1 μM carbamoylcholine bind [^{125}I]αBuTx at a substantially slower rate, indicating a conversion from the low-affinity state for the agonist to the high-affinity one (Weiland et al., 1977; Quast et al., 1978). In the experiment shown here, the conversion to high affinity is less complete with membranes receiving the dual treatment. In other experiments with the same membranes, the conversion was indistinguishable from that of normal membranes. The high-affinity form of the membrane-bound receptor is stabilized by local anesthetics (Cohen et al., 1980) and Triton X-100 (Brisson et al., 1975) and by treatment with phospholipase A (Andreasen & McNamee, 1977). Volatile anesthetics facilitate conversion to the desensitized form (Young et al., 1978) while solubilization of the receptor drastically alters the transition (Weiland et al., 1976). Thus, the ability of the receptors to undergo this conformational change is a sensitive test of perturbation. These results argue against any

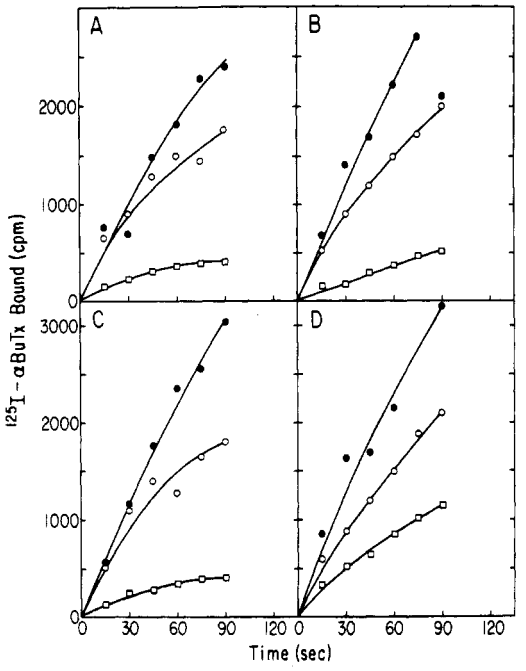

FIGURE 3: Binding of [^{125}I]αBuTx to (A) normal membranes, (B) saponin-treated membranes, (C) alkaline-extracted membranes, and (D) saponin-treated, alkaline-extracted membranes in the absence of carbamoylcholine (●), in the presence of 1 μM carbamoylcholine added simultaneously with the toxin (○), or after preincubation of the membranes with 1 μM carbamoylcholine for 15 min before the addition of toxin (□). All reactions were performed at 25 °C.

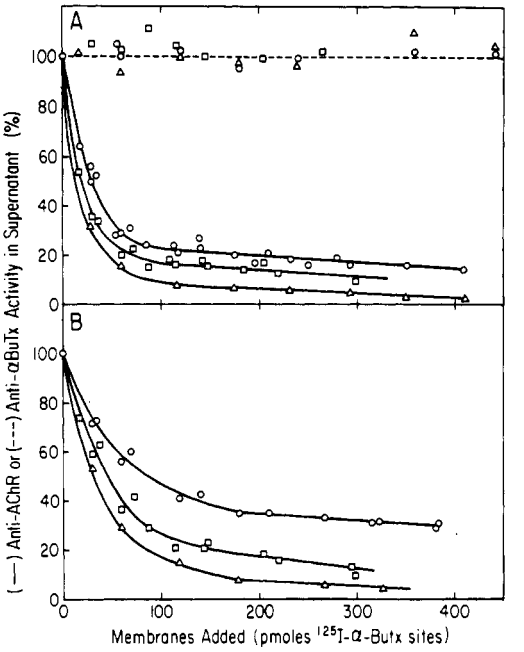

FIGURE 4: Binding of (A) anti-nAChR 1 (—) and anti-αBuTx (---) and (B) anti-nAChR 2 to normal membranes (○), saponin-treated membranes (□), or alkaline-extracted membranes (△). Binding was measured by absorption of 5 μL of anti-nAChR 1, 10 μL of anti-nAChR 2, or 10 μL of anti-αBuTx in a total volume of 200 μL with the indicated amounts of membranes, followed by determination of the antibody activity remaining in the supernatant as described under Experimental Procedures.

substantial alteration in receptor structure as a result of the treatments. It should also be noted that concentrations of saponin at least 30-fold higher than those used to permeabilize the vesicles are completely ineffective in solubilizing the receptor (data not shown).

Binding of Antibodies to the Membrane-Bound Receptor. Attempts to measure the binding of antibodies to receptor-rich membranes directly using [^{125}I]IgG were unsuccessful because of high levels of nonspecific binding of radioiodinated antibodies (C. McKay and S. C. Froehner, unpublished experiments). Therefore, we developed a procedure in which antisera were incubated with membrane vesicles, the immune complexes were removed by centrifugation, and the amount of anti-AChR activity remaining in the supernatant was determined quantitatively as described above. This procedure detects antibodies directed against sites on detergent-solubilized AChR that are inaccessible in the membrane-bound receptor.

Both anti-nAChR 1 and anti-nAChR 2 bind well to normal membranes. As shown in Figure 4, untreated membranes, when present in excess, are capable of binding at least 80% of the antibodies in anti-nAChR 1 and about 70% of anti-nAChR 2. Two lines of evidence indicate that this binding is specific. First, a control antiserum, rabbit anti-αBuTx, does not bind to the membranes (Figure 4). Second, the anti-AChR antibodies do not bind to human erythrocyte membranes or to partially purified from *Electrophorus* electic tissue when used at similar membrane protein concentrations (data not shown). *Electrophorus* contains much lower levels of AChR than does *Torpedo*, and they show only partial cross-reactivity with anti-*Torpedo* AChR antisera (Lindstrom et al., 1979b). In contrast to the antisera to native AChR, both anti-dAChR sera bind poorly to normal membranes (Figure 5). In this experiment, only 10–20% of both anti-dAChR 1 and anti-dAChR 2 could be bound to the membranes. Thus, these antisera recognize predominantly antigenic determinants that are inaccessible to antibodies when the receptor is membrane

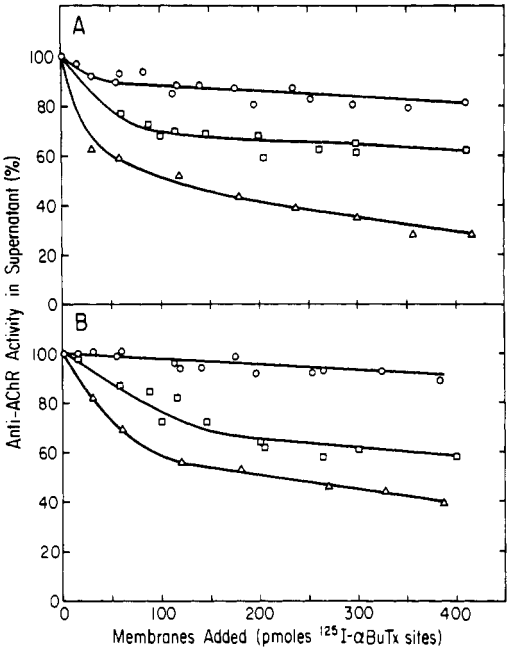

FIGURE 5: Binding of (A) anti-dAChR 1 and (B) anti-dAChR 2 to normal membranes (○), saponin-treated membranes (□), or alkaline-extracted membranes (△). Absorption were carried out with 10 μL of anti-dAChR 1 and 20 μL of anti-dAChR 2 as described under Experimental Procedures.

bound. Preincubation of the membranes with excess αBuTx or with carbamoylcholine did not detectably affect the binding of anti-dAChR 1 or anti-nAChR 2.

Some of the inaccessible sites can be exposed by either saponin permeabilization or alkaline extraction of the membrane vesicles. Saponin treatment increases the binding of anti-dAChR 1 and anti-dAChR 2 2- and 4-fold, respectively. Alkaline extraction increases anti-dAChR 1 binding 3–4-fold and anti-dAChR 2 binding 5–6-fold (Figure 5). The effects

Table I: Absorption of Antisera with AChR-Rich Membranes[a]

antiserum	antibody activity remaining (% ± SD)			
	normal	saponin treated	alkaline extracted	saponin treated + alkaline extracted
anti-nAChR 1	16 ± 5 (12)	13 ± 6 (6)	4 ± 2 (8)	2 ± 3 (3)
anti-nAChR 2	28 ± 8 (12)	14 ± 6 (7)	7 ± 4 (6)	3 ± 4 (3)
anti-dAChR 1	79 ± 12 (18)	52 ± 10 (10)	38 ± 12 (11)	19 ± 9 (4)
anti-dAChR 2	82 ± 9 (18)	48 ± 10 (10)	40 ± 8 (10)	19 ± 11 (4)
anti-δ chain	78 ± 16 (8)	40 ± 7 (3)	32 ± 8 (7)	10 ± 5 (3)
anti-αBuTx	107 ± 5 (4)	98 ± 4 (4)	110 ± 7 (4)	112 ± 10 (4)

[a] Values in parentheses are the number of determinations. Binding was performed with concentrations of membranes shown to be in excess (300–400 pmol of [^{125}I]αBuTx sites).

of alkaline extraction and saponin treatment on binding are much smaller when the antisera to native AChR are used, mainly because they recognize predominantly sites that are accessible in the membrane-bound receptor. Nevertheless, small but reproducible increases in binding are seen for both antisera (Figure 4). Neither alkaline extraction nor saponin treatment promotes nonspecific binding of anti-αBuTx (Figure 4).

In alkaline-extracted membranes, the anti-dAChR sera appear to recognize two classes of sites, one present in relatively high concentration (or with high affinity for the antibodies) and one present in relatively low concentration (or with low affinity). As a result, saturation of antibody binding is not obtained, even at 400 pmol of [^{125}I]αBuTx sites. Since alkaline-extracted membrane preparations contain both sealed and unsealed vesicles, it is possible that the two populations of sites represent newly exposed extracellular as well as intracelllar determinants. The concentration of accessible intracellular sites could be low, depending on the proportion of vesicles that remain sealed. Treatment of alkaline-extracted membranes with saponin should permeabilize all of the vesicles and permit binding of antibodies to intravesicular sites that are normally masked by peripheral proteins. As shown in Table I, membranes subjected to the combined treatment are very effective in binding antibodies.

The results in Table I represent a summary of binding experiments performed with membranes prepared from three fish. A total of eight preparations of alkaline-extracted membranes, six preparations of saponin-treated membranes, and three preparations of membranes receiving both treatments were studied. Alkaline extraction was carried out at either 4 or 25 °C. The binding to membranes extracted at 25 °C was in general slightly higher. The results are in general agreement with those shown in Figures 4 and 5. In addition, it is clear that the alkaline-extracted membranes treated with saponin are the most effective in binding antibodies. Normal membranes bind only 10–20% of the antibodies to the denatured receptor or its δ subunit while dual-treated membranes binding 80–90%. Thus, virtually all determinants recognized by five different antisera, many of which are inaccessible in the membrane-bound receptor, can be exposed by removal of peripheral proteins from and permeabilization of the membrane vesicles.

One possible explanation for the differential binding of the anti-dAChR and anti-nAChR sera to membrane-bound receptors is that they recognize different subunits of the receptor. We examined this possibility by separating the receptor subunits by $NaDodSO_4$ microslab gel electrophoresis and then reacting the antisera with the proteins in the gel. After removal of unbound proteins, the bound antibodies were localized by reaction with ^{125}I-labeled protein A and autoradiography after extensive washing. For demonstration of the specificity

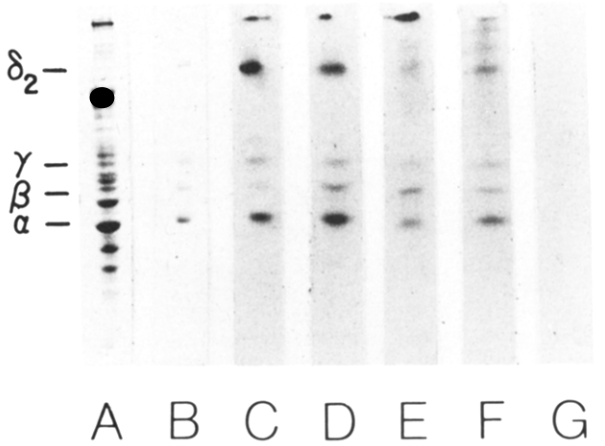

FIGURE 6: Reaction of antisera with electroplax proteins after $NaDodSO_4$ gel electrophoresis. Gels of (A) total electroplax cellular protein and (B) purified AChR stained for protein with Coomassie Blue. (C–G) Autoradiograms of gels of total cellular protein [same sample as (A)] after incubation with (C) anti-dAChR 1, (D) anti-dAChR 2, (E) anti-nAChR 1, (F) anti-nAChR 2, and (G) anti-αBuTx followed by reaction with ^{125}I-labeled protein A. Greek letters to the left show positions of AChR subunits in (B). Since samples were not reduced prior to electrophoresis, the δ chain migrates as a dimer (δ_2). δ_2 migrates more slowly than the component of ~90 000 molecular weight which is a minor contaminant of some AChR preparations.

of the technique, total cellular electroplax protein, of which the ACh receptor comprises less than 0.5%, was solubilized in $NaDodSO_4$ in the presence of protease inhibitors and then subjected to gel electrophoresis. As shown in Figure 6, all of the anti-AChR sera contain antibodies which bind to all four receptor subunits. In addition, a small amount of binding of anti-nAChR 2 to high molecular weight polypeptides is seen. Anti-αBuTx fails to bind any of the polypeptides.

The same procedure, utilizing affinity-purified AChR as the antigen, was used to determine on which subunit(s) the buried antigenic determinants are located. Absorption of anti-dAChR 1 with normal membranes removes antibodies specific for the α subunit (Figure 7C), as indicated by a significant reduction in the intensity of the labeling of α compared to that of unabsorbed antiserum (Figure 7B). Saponin-treated membranes can bind additional antibodies specific for the α- as well as the δ-chain (Figure 7D). Alkaline extraction exposes sites on α and δ as well (Figure 7E). Finally, the combination of saponin treatment and alkaline extraction uncovers determinants on all four subunits not accessible in normal membranes (Figure 7F). The intensity of the bands on the autoradiogram correlated well with the amount of radioactivity (as measured directly by γ counting) associated with them in the gel. Virtually indentical results are found with anti-dAChR 2. Reaction with normal membranes removes anti-α-chain antibodies (Figure 7I) while either saponin-treated (Figure 7J)

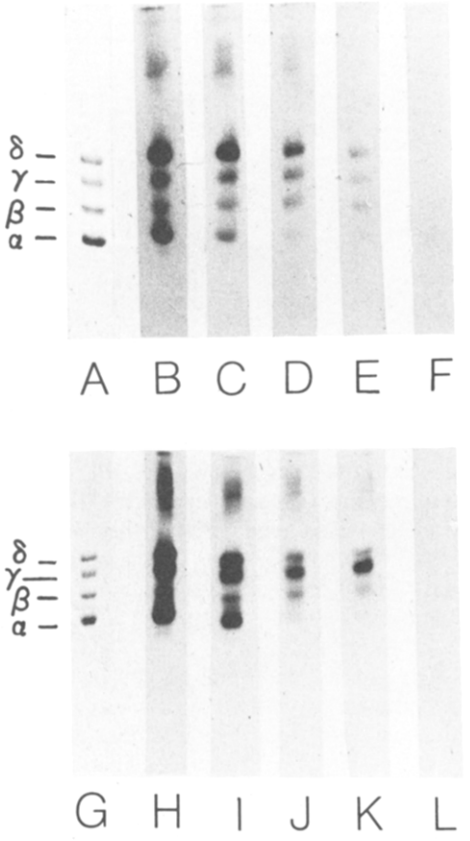

FIGURE 7: Reactivity of absorbed anti-dAChR sera with AChR subunits after $NaDodSO_4$ gel electrophoresis. (A and G) Gels of purified AChR stained for protein with Coomassie Blue. Autoradiograms of gels reacted with (B–F) anti-dAChR 1 or (H–L) anti-dAChR 2 after absorption with (B and H) no membranes, (C and I) normal membranes, (D and J) saponin-treated membranes, (E and K) alkaline-extracted membranes, or (F and L) saponin-treated, alkaline-extracted membranes, followed by reaction with ^{125}I-labeled protein A.

or alkaline-extracted membranes (Figure 7K) bind antibodies specific for α and δ. Combined treatment exposes latent sites on all four subunits (Figure 7L), resulting in the removal of all activity detectable by this method.

The antibody prepared to purified δ chain reacts with both the γ and the δ chain (Figure 8B). Similar results have been reported with antisera prepared in rats against individual receptor chains (Lindstrom et al., 1979b) as well as with several monoclonal antibodies (Tzartos & Lindstrom, 1980). In view of the extensive amino acid sequence homology of the first 56 amino-terminal residues of the γ and δ subunits (Raftery et al., 1980), immunological cross-reactivity of these two polypeptides is not unexpected. Although the quantitative precipitation assay demonstrated that normal and saponin-treated membranes bind some of the antibodies in this serum (Table I), little difference is seen in the extent of subunit binding in $NaDodSO_4$ gels by anti-δ absorbed with these membranes compared to unabsorbed serum (Figure 8C,D). Alkaline-extracted membranes bind antibodies specific for both γ and δ (Figure 8E), as do permeabilized, stripped membranes (Figure 8F).

Since normal membranes bind most of the antibodies present in anti-nAChR 1 and anti-nAChR 2, absorbed sera show little reactivity with subunits. Absorption of anti-nAChR 1 with normal membranes removes all antibodies except some specific for the γ chain (Figure 9C). These do not bind to saponin-treated membranes (Figure 9D) but do to alkaline-extracted vesicles (Figure 9E). Similarly, anti-nAChR 2 reacts with α, β, and δ in normal membranes (Figure 9I). The binding to

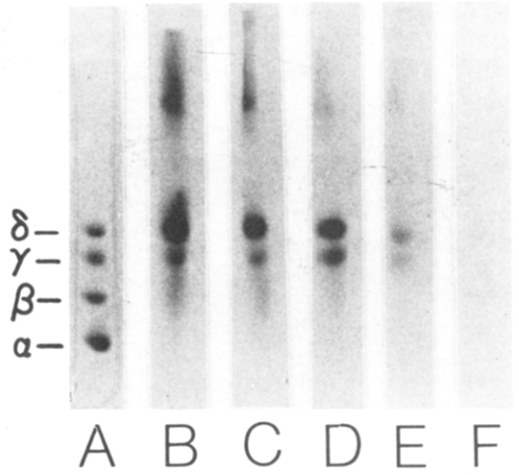

FIGURE 8: Reactivity of absorbed anti-δ-chain serum with AChR subunits after $NaDodSO_4$ gel electrophoresis. Coomassie Blue stained gel (A) of purified AChR and autoradiograms (B–F) of gels of purified AChR reacted with (B) unabsorbed anti-δ serum or with anti-δ absorbed with (C) normal membranes, (D) saponin-treated membranes, (E) alkaline-extracted membranes, or (F) saponin-treated, alkaline-extracted membranes, followed by reaction with ^{125}I-labeled protein A.

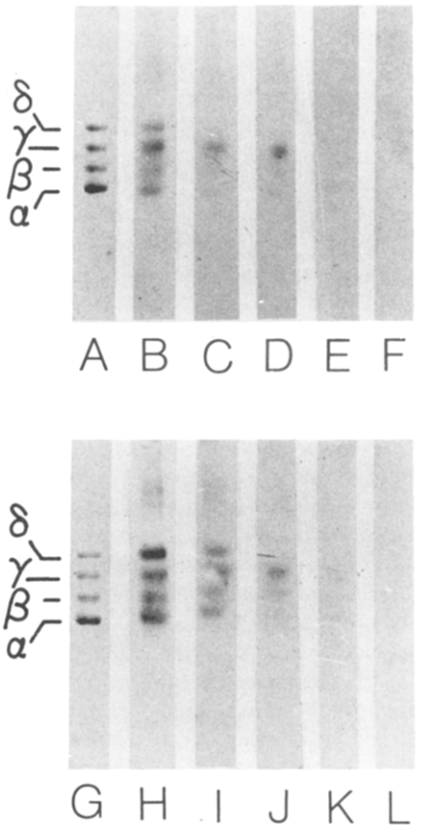

FIGURE 9: Ractivity of absorbed anti-nAChR sera with AChR subunits after $NaDodSO_4$ gel electrophoresis. Coomassie Blue stained gels (A and G) of purified AChR and autoradiograms of gels purified AChR after reaction with (B–F) anti-nAChR 1 or (H–L) anti-nAChR 2 followed by ^{125}I-labeled protein A. Antisera were absorbed with (B and H) no membranes, (C and I) normal membranes, (D and J) saponin-treated membranes, (E and K) alkaline-extracted membranes, or (F and L) saponin-treated, alkaline-extracted membranes.

α and δ is increased in saponin-treated membranes (Figure 9J) and to α, γ, and δ in alkaline-extracted vesicles (Figure 9K).

A summary of the subunit location of exposed and buried antigenic determinants recognized by the five antisera is given in Table II. With the possible exception of the exposure of

Table II: Subunit Location of Antigenic Determinants Exposed by Membrane Treatments

	treatment and subunits															
	none				saponin[a]				alkaline extraction[a]				dual treatment[b]			
antisera	α	β	γ	δ	α	β	γ	δ	α	β	γ	δ	α	β	γ	δ
anti-nAChR 1	+	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−
anti-nAChR 2	+	+	−	+	+	−	−	+	+	−	+	+	−	−	−	−
anti-dAChR 1	+	−	−	−	+	−	−	+	+	−	−	+	−	+	+	(+)
anti-dAChR 2	+	−	−	−	+	−	−	+	+	−	−	+	−	(+)	+	+
anti-δ	−	−	−	−	−	−	−	−	−	−	(+)[c]	+	−	−	(+)	+

[a] Compared to untreated membranes. [b] Compared to membranes receiving either treatment. [c] Displayed a small differential binding to treated membranes, and result is therefore uncertain.

sites on β by the combined treatment, each result is verified by more than one antiserum. Subunits α, β, and δ possess determinants exposed in untreated vesicles. Buried determinants are detected on each of the subunits. Some of those located on α and δ can be exposed by saponin permeabilization, while alkaline extraction uncovers new determinants on α, γ, and δ. A combination of the two treatments uncovers sites on β, γ, and δ that neither treatment alone exposes.

Discussion

The structure of the membrane-bound ACh receptor from *Torpedo* has been investigated by X-ray diffraction (Ross et al., 1977) and by electron microscopy in negatively stained preparations (Klymkowsky & Stroud, 1979) and after reaction with antireceptor antibodies coupled to ferritin (Karlin et al., 1978). These studies indicate that the receptor complex extends out from the lipid bilayer 50–55 Å on the extracellular side and much less (~15 Å) on the intracellular face. Therefore, it is not unexpected that antibodies prepared to solubilized, affinity-purified receptor recognize the membrane-bound receptor in right-side-out, sealed vesicles. Several morphological studies have demonstrated the binding of anti-AChR antibodies to receptor-rich membrane vesicles (Tarrab-Hazdai et al., 1978; Karlin et al., 1978; Strader et al., 1979; Klymkowsky & Stroud, 1979; St. John et al., 1979). The methods that we have employed are more quantitative than the morphological techniques and indicate that 80–90% of the antigenic sites recognized by anti-nAChR sera are extracellularly disposed. The anti-dAChR sera also recognize extracellular determinants but to a much smaller extent. Visualization of the binding of antibodies prepared against denatured AChR or denatured subunits using ferritin or hemocyanin-conjugated antibodies failed to detect binding to membrane-bound receptors (Karlin et al., 1978; Strader et al., 1979). Thus, the absorption technique used here may be much more sensitive than the morphological ones.

Three of the four polypeptide chains of the AChR possess extracellular antigenic sites that are recognized by the five antisera that we have studied. Absorption of the antisera with untreated membranes removed antibodies reactive with the α, β, and δ polypeptides. These results are consistent with other results which indicate that all four chains are extracellularly disposed. All are glycosylated (Vandlen et al., 1979; Lindstrom et al., 1979a), and three (α, β and δ) can be labeled by lactoperoxidase-catalyzed radioiodination under conditions in which the interior of vesicles is inaccessible (P. St. John, J. B. Cohen, and D. A. Goodenough, unpublished experiments). Photoactivable derivatives of αBuTx react with all four chains of the receptor in *Torpedo* membranes (Witzemann & Raftery, 1978; Nathanson & Hall, 1980), presumably on the extracellular surface. In addition, all four subunits are sensitive to proteolysis from the exterior (Strader & Raftery, 1980). Failure to detect extracellular determinants on the γ chain suggests that this polypeptide may be exposed to only a limited extent on the outside of the vesicles or that the extracellular portion of this chain is not very antigenic in rabbits, or both.

In addition to the extracellular sites, all five antisera recognize determinants that are inaccessible when the receptor is membrane bound. The majority of the sites recognized by the anti-dAChR sera are hidden. Furthermore, it is clear that each of the subunits has buried determinants. Buried sites are detected on α, γ, and δ by at least three different antisera and on β by two antisera.

Some of the buried determinants can be exposed to antibodies by permeabilization of the vesicles with saponin. Results with three different antisera demonstrate that saponin treatment exposes new sites on the α and δ subunits. Although we cannot entirely exclude the possibility that saponin exposes extracellular sites by altering the conformation of the receptor, this seems unlikely. Saponin, which is known to interact with cholesterol (Lucy & Glaubert, 1964), is ineffective in solubilizing the receptor, even at concentrations 30 times that used to permeabilize the vesicles. Furthermore, after saponin treatment, the receptors still undergo agonist-induced affinity changes indistinguishable from that of untreated membranes. The major effect of 0.1% saponin treatment is to convert the receptor-containing vesicles from a state of approximately 90% impermeable to macromolecules to one of 100% permeable (St. John et al., 1979). After treatment, the vesicles remain in a right-side-out orientation (St. John et al., 1979). Therefore, it seems reasonable to conclude that saponin treatment exposes receptor determinants that are cytoplasmically disposed and/or located within the lipid bilayer. By this criterion, then, α and δ are transmembrane polypeptides.

Alkaline extraction of the receptor-rich membranes also exposes a large number of buried determinants. This treatment removes a major polypeptide of 43 000 daltons as well as others of 50 000–55 000 and ~90 000 daltons without affecting several aspects of receptor function. After alkaline extraction, however, the membrane-bound receptor is more sensitive to trypsin digestion (Klymkowsky et al., 1980) and heat inactivation (Saitoh et al., 1979). Furthermore, the vesicles have a smaller internal volume (Neubig et al., 1979) and a lower density (Klymkowsky et al., 1980). In untreated membranes, the rotational mobility of the receptor is highly restricted (Rousselet & Devaux, 1977; Lo et al., 1980). Alkaline extraction greatly increases the rotational mobility (Rousselet et al., 1979; Lo et al., 1980). Also, the receptor rosettes seen in negatively stained preparations are more widely spaced in alkaline-treated membranes (Barrantes et al., 1980). Finally, alkaline extraction allows macromolecular access to the interior of at least some vesicles. The extent of permeabilization is variable but generally much less than that caused by saponin (St. John, 1980).

The basis for exposure of antigenic sites by alkaline extraction, therefore, may be 2-fold. Extracellular determinants

normally inaccessible because of interactions between receptor complexes or between the complexes and peripheral proteins, for example, may be uncovered by this treatment. A variable number of cytoplasmically disposed determinants, including ones that interact with peripheral membrane proteins, may also become exposed, depending on the extent of permeabilization. Binding of anti-dAChR to alkaline-extracted membranes provides evidence for two general classes of sites, one present in relatively high concentrations and one much less prevalent. Although the topology of these sites is unclear, they are present on the α, γ, and δ chains.

The augmentation by saponin of antibody binding to alkaline-extracted membranes is most likely due to permeabilization of a population of sealed, alkaline-treated vesicles. Since this category of sites (found on the β, γ, and δ subunits) is not exposed by either saponin treatment or alkaline extraction alone, they probably represent intracellular sites that are normally masked by receptor–peripheral protein interactions. These results provide evidence that all four subunits possess cytoplasmically disposed determinants. Thus, all appear to be transmembrane.

Although the receptor is thought to be transmembrane protein, this has been difficult to demonstrate by immunohistochemical techniques. Immunoferritin techniques have detected intravesicular labeling of the ACh receptor only rarely when vesicles were not permeabilized (Tarrab-Hazdai et al., 1978; Karlin et al., 1978). In one case, it was suggested that these rare observations were not readily distinguished from nonspecific trapping of antibodies or ferritin (Karlin et al., 1978). Even in saponin-permeabilized vesicles, intravesicular determinants have not been detected by using ferritin-conjugated antibodies in a sandwich technique (St. John, 1980). Strader et al. (1979) exposed the cytoplasmic face of vesicles by attaching them to cover slips and then shearing off the unattached portion by sonication. With protein A-hemocyanin as the marker, one out of six antisera investigated labeled cytoplasmically disposed sites. All of the antisera used for these studies were prepared against native AChR and, therefore, may be directed primarily against extracellular sites. However, attempts to detect intravesicular sites in saponin-permeabilized vesicles with anti-dAChR 1 and anti-dAChR 2 using immunoferritin techniques have not been successful (St. John, 1980). In fact, no ferritin labeling was observed on either side of the membrane with these two antisera, even after alkaline extraction of permeabilized vesicles. This technique may lack the sensitivity required to detect intracellular sites. Alternatively, binding of the large ferritin–second antibody to antireceptor antibodies bound to the membrane may be sterically hindered.

Recent biochemical evidence suggests that all four chains of the receptor are transmembrane. Wennogle & Changeux (1980) found that receptor-rich membrane proteins of 40 000, 50 000, and 66 000 daltons were highly resistant to trypsin digestion unless the vesicles were pretreated at pH 4 or at 62 °C. A polypeptide of 43 000 molecular weight was unaffected, even after pretreatment. Sonication of the vesicles in the presence of trypsin, thought to allow proteolytic attack from the interior, resulted in degradation of all four of the polypeptides. Since their preparations lack the γ polypeptide, no information about its proteolytic sensitivity was obtained. They concluded that three of the receptor chains (presumably α, β, and δ) are transmembrane and that the 43 000-dalton peripheral protein is located on the cytoplasmic face. Strader & Raftery (1980) used a freeze–thaw method to introduce trypsin to the interior of alkaline-extracted vesicles (presumably that portion of the vesicle population that remains sealed after extraction) and found that all four receptor chains can be proteolyzed from either the inside or the outside of the vesicles. The lipophilic photolabel [^{3}H]pyrenesulfonyl azide reacts with the β and γ chains of the membrane-bound receptor (Sator et al., 1979) while 5-[^{125}I]iodonaphthyl 1-azide labels only the α chain (Tarrab-Hazdai et al., 1980), indicating that all three of these chains are associated with the hydrophobic portion of the lipid bilayer. Our findings, suggesting that all four of the reeptor subunits are transmembrane, are consistent with these results.

The inability of anti-dAChR to bind to AChR in membranes may account for the failure of rabbits immunized with denatured AChR to develop EAMG (Bartfeld & Fuchs, 1977). Antisera that bind the membrane-associated receptor poorly may be unable to promote antigenic modulation (Heinemann et al., 1978; Reiness et al., 1978; Stanley & Drachman, 1978), initiate complement fixation (Lennon et al., 1978), or affect AChR function directly (Patrick et al., 1973; Lindstrom et al., 1976; Karlin et al., 1978). However, additional factors may also be important. In a thorough study of different strains of mice, Berman & Patrick (1980) found a poor correlation between antibody titers against solubilized AChR and induction of the experimental disease. Some antisera from mice with no clinical symptoms were capable of increasing the degradation rate of AChR in BC_3H-1 cells. At least in mice, the ability of an antibody population to promote a defect in neuromuscular transmission is not necessarily a direct result of its ability to recognize the membrane-bound receptor.

It would be of interest to examine human myasthenic serum by the methods developed here for the presence of antibodies that recognize buried determinants of the receptor. Little is known about the immunogen that triggers the autoimmune response. The presence of antibodies against buried determinants would suggest that the immunogen is shed from the membrane at some stage in the induction. Because techniques for isolating the membrane-bound mammalian receptor have not been developed, such studies would necessarily be restricted to human antibodies that cross-react with the *Torpedo* receptor.

Acknowledgments

I thank Vivian Gulbrandsen for expert technical assistance and Charles McKay for performing some of the initial experiments. I also thank Drs. Jonathan Cohen and Paul St. John for their advice concerning the membrane preparations.

References

Adair, W. S., Jurivich, D., & Goodenough, U. W. (1978) *J. Cell Biol. 79*, 281–285.

Andreasen, T. J., McNamee, M. G. (1977) *Biochem. Biophys. Res. Commun. 79*, 958–965.

Barrantes, F. J., Neugebauer, D.-Ch., & Zingsheim, H. P. (1980) *FEBS Lett. 112*, 73–78.

Bartfeld, D., & Fuchs, S. (1977) *FEBS Lett. 77*, 214–218.

Berman, P. W., & Patrick, J. (1980) *J. Exp. Med. 151*, 204–223.

Blakesley, R. W., & Boezi, J. A. (1977) *Anal. Biochem. 82*, 580–582.

Brisson, A., Devaux, P. F., & Changeux, J.-P. (1975) *C. R. Hebd. Seances Acad. Sci., Ser. D 280*, 2153–2156.

Burridge, K. (1976) *Proc. Natl. Acad. U.S.A. 73*, 4457–4461.

Chang, H. W., & Bock, E. (1977) *Biochemistry 16*, 4513–4520.

Claudio, T., & Raftery, M. (1977) *Arch. Biochem. Biophys. 181*, 484–489.

Cohen, J. B., Boyd, N. D., & Shera, N. S. (1980) in *Molecular Mechanisms of Anesthesia* (Fink, B. R., Ed.) pp 165–174, Raven Press, New York.

Damle, V. N., McLaughlin, M., & Karlin, A. (1978) *Biochem. Biophys. Res. Commun. 84*, 845–851.

Daniels, M. P., & Vogel, Z. (1978) *Princ. Tech. Electron Microsc. 9*, 107–125.

Elliot, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P., Racs, J., & Raftery, M. A. (1980) *Biochem. J. 185*, 667–677.

Epstein, M., & Racker, E. (1978) *J. Biol. Chem. 253*, 6660–6662.

Froehner, S. C., & Rafto, S. (1979) *Biochemistry 18*, 301–307.

Froehner, S. C., Reiness, C. G., & Hall, Z. W. (1977) *J. Biol. Chem. 252*, 8589–8596.

Froehner, S. C., Gulbrandsen, V., Hyman, C., Neubig, R. R., Jeng, A. Y., & Cohen, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.

Heinemann, S., Merlie, J. P., & Lindstrom, J. (1978) *Nature (London) 274*, 65–68.

Hubbard, A. L., & Cohn, Z. A. (1975) *J. Cell Biol. 64*, 438–460.

Hucho, F., Layer, P., Kiefer, H. R., & Bandini, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2624–2628.

Karlin, A. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1847–1853.

Karlin, A. (1980) in *The Cell Surface and Neuronal Function* (Cotman, C. W., Poste, G., & Nicolson, G. L., Eds.) pp 191–260, Elsevier/North-Holland Biomedical Press, Amsterdam.

Karlin, A., Holtzman, E., Valderrama, R., Damle, V., & Reyes, F. (1978) *J. Cell Biol. 76*, 577–592.

Klymkowsky, M. W., & Stroud, R. M. (1979) *J. Mol. Biol. 128*, 319–334.

Klymkowsky, M. W., Heuser, J. E., & Stroud, R. M. (1980) *J. Cell Biol. 85*, 823–838.

Lennon, V. A., Seybold, M. E., Lindstrom, J., Cochrane, C., & Yulevitch, R. (1978) *J. Exp. Med. 147*, 973–983.

Lindstrom, J. (1979) *Adv. Immunol. 27*, 1–50.

Lindstrom, J., Lennon, V., Seybold, M., & Whittingham, S. (1976) *Ann. N.Y. Acad. Sci. 274*, 254–274.

Lindstrom, J., Einarson, B., & Merlie, J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 769–773.

Lindstrom, J., Merlie, J., & Yogeeswaran, G. (1979a) *Biochemistry 18*, 4465–4469.

Lindstrom, J., Walter (Nave), B., & Einarson, B. (1979b) *Biochemistry 18*, 4470–4480.

Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osame, M., & Montal, M. (1980a) *J. Biol. Chem. 255*, 8340–8350.

Lindstrom, J., Gullick, W., Conti-Tronconi, B., & Ellisman, M. (1980b) *Biochemistry 19*, 4791–4795.

Lo, M. M. S., Garland, P. B., Lamprecht, J., & Barnard, E. A. (1980) *FEBS Lett. 111*, 407–412.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lucy, J. A., & Glaubert, A. M. (1964) *J. Mol. Biol. 8*, 727–748.

Matsudaira, D., & Burgess, D. (1978) *Anal. Biochem. 87*, 386–396.

Moore, H.-P. H., & Raftery, M. A. (1979) *Biochemistry 18*, 1862–1867.

Moore, H.-P. H., Hartig, P. R., & Raftery, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6265–6269.

Nathanson, N. M., & Hall, Z. W. (1979) *Biochemistry 18*, 3392–3401.

Nathanson, N. M., & Hall, Z. W. (1980) *J. Biol. Chem. 255*, 1698–1703.

Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.

Patrick, J., & Lindstrom, J. (1973) *Science (Washington, D.C.) 180*, 871–872.

Patrick, J., Lindstrom, J., Culp, B., & McMillan, J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3334–3338.

Quast, U., Schimerlik, M., Lee, T., Witzeman, V., Blanchard, S., & Raftery, M. A. (1978) *Biochemistry 17*, 2405–2414.

Raftery, M. A., Schmidt, J., & Clark, D. G. (1972) *Arch. Biochem. Biophys. 152*, 882–886.

Raftery, M. A., Hunkapiller, M. W., Strader, C. R., & Hood, L. E. (1980) *Science (Washington, D.C.) 208*, 1454–1456.

Reiness, C. G., Weinberg, C., & Hall, Z. W. (1978) *Nature (London) 274*, 68–70.

Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.

Ross, M. J., Klymkowsky, M. W., Agard, D. A., & Stroud, R. M. (1977) *J. Mol. Biol. 116*, 635–659.

Rousselet, A., & Devaux, P. F. (1977) *Biochem. Biophys. Res. Commun. 78*, 448–454.

Rousselet, A., Cartaud, J., & Devaux, P. F. (1979) *C. R. Hebd. Seances Acad. Sci., Ser. D 289*, 461–463.

Saitoh, T., Wennogle, L. P., & Changeux, J.-P. (1979) *FEBS Lett. 108*, 489–494.

Sator, V., Gonzales-Ros, J. M., Calvo-Fernadez, P., & Martinez-Carrion, M. (1979) *Biochemistry 18*, 1200–1206.

Sobel, A., Weber, M., & Changeux, J.-P. (1977) *Eur. J. Biochem. 80*, 215–224.

Stanley, E., & Drachman, D. (1978) *Science (Washington, D.C.) 200*, 1285–1287.

St. John, P. (1980) Ph.D. Thesis, Harvard University, Cambridge, MA.

St. John, P., Cohen, J. B., & Goodenough, D. A. (1979) *Soc. Neurosci. Abstr. 5*, 309.

Strader, C. D., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5807–5811.

Strader, C. D., Revel, J.-P., & Raftery, M. A. (1979) *J. Cell Biol. 83*, 499–510.

Tarrab-Hazdai, R., Geiger, B., Fuchs, S., & Amsterdam, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2497–2501.

Tarrab-Hazdai, R., Bercovici, T., Goldfarb, V., & Gitler, C. (1980) *J. Biol. Chem. 255*, 1204–1209.

Tzartos, S. J., & Lindstrom, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.

Valderrama, R., Weill, C. L., McNamee, M. G., & Karlin, A. (1976) *Ann. N.Y. Acad. Sci. 274*, 108–115.

Vandlen, R. L., Wu, W. C.-S., Eisenach, J. C., & Raftery, M. A. (1979) *Biochemistry 18*, 1845–1854.

Vogel, Z., Sytkowski, A., & Nirenberg, M. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3180–3184.

Weiland, G., Georgia, B., Wee, V. T., Chignell, C. F., & Taylor, P. (1976) *Mol. Pharmacol. 12*, 1091–1105.

Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., & Taylor, P. (1977) *J. Biol. Chem. 252*, 7648–7656.
Weiland, G., Frisman, D., & Taylor, P. (1979) *Mol. Pharmacol. 15*, 213–226.
Weill, C. L., McNamee, M. G., & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997–1003.
Wennogle, L. P., & Changeux, J.-P. (1980) *Eur. J. Biochem. 106*, 381–393.
Witzemann, V., & Raftery, M. (1978) *Biochem. Biophys. Res. Commun. 81*, 1025–1031.
Young, A. P., Brown, F. F., Halsey, M. J., & Sigman, D. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4563–4567.

Penetration of Phospholipid Monolayers by Cardiotoxins†

Pierre Bougis,* Hervé Rochat, Gérard Piéroni, and Robert Verger

ABSTRACT: The monomolecular film technique was used to compare the specific interactions of four cardiotoxins from *Naja mossambica mossambica* with different phospholipids. We were able to demonstrate the interaction of cardiotoxins (10^{-7} M) with both neutral and negatively charged phospholipids up to very high surface pressures (45 dyn/cm). In the presence of a phospholipid monolayer, the surface activity of cardiotoxins became much greater than that observed at the air–water interface. Neurotoxins of the same venom do not penetrate a phospholipid film, even at low surface pressure (15 dyn/cm). The apparent molecular area of cardiotoxin III during its insertion into a negatively charged phospholipid film was quantitatively defined. As a function of surface pressure of the membrane around 25 dyn/cm, cardiotoxins may exist in two different configurations, "flat" (1400 Å^2) or "edgewise" (420 Å^2). This result could account for the lytic activity of this type of toxin.

Certain snake venoms, principally those of cobras (*Elapidae*), contain toxic proteins lacking enzymatic activity. Among these toxins, polycationic molecules called cardiotoxins (Sarkar, 1947), cytotoxins (Braganca et al., 1967; Patel et al., 1969), cobramines (Larsen & Wolff, 1968), or direct lytic factors (Condrea et al., 1964; Aloof-Hirsch et al., 1968) constitute a family of homologous proteins, generally composed of 60 amino acid residues (about 7000 daltons) and reticulated by four disulfide bridges.

Although interactions other than protein–lipid have been suggested (Vogt et al., 1970; Lin et al., 1975–1977), protein–lipid interactions are apparently involved in the fixation of cardiotoxins to the cell membrane since purified phospholipids inhibit their action with intact cells and reverse the inactivation of Na^+/K^+ dependent ATPase (Patel et al., 1969; Zaheer et al., 1975). Furthermore, studies on the fixation of tritiated cardiotoxin derivatives on axon membranes suggest a direct association of the toxin with lipid-type receptor structure since the number of fixation sites was found to be higher than the total number of membrane proteins (Vincent et al., 1976).

The direct study of cardiotoxin–lipid interactions thus seems to be a useful experimental approach toward the understanding of the biological effects of these molecules at the membrane level, especially concerning cellular lysis (Yang, 1974; Condrea, 1974). Studies of the variation of the intrinsic fluorescence of Trp_{11} of certain of these toxins led to the demonstration of their reversible fixation to bilamellar phospholipid vesicles (liposomes), which was inhibited by an ionic strength or pH effect (Dufourcq & Faucon, 1978; Vincent et al., 1978). The lipid–toxin complex with a stoichiometry of 7:1 lipid molecules/toxin molecule can apparently be formed only with phopholipids which are negatively charged at neutral pH ($K_d < 10^{-6}$ M).

The present report describes the use of the monomolecular film technique for the analysis of the specificity of interactions between the cardiotoxins of *Naja mossambica mossambica* and phospholipids. The apparent molecular area of these toxins during their insertion into a lipid film was quantitatively defined. A comparative study was performed simultaneously with neurotoxins from the same snake.

Materials and Methods

Toxins. The four cardiotoxins contained in the venom of *Naja mossambica mossambica* were purified in our laboratory. The fraction containing cardiotoxin activity obtained by filtration of the venom through Sephadex G-50 in 0.1 M ammonium acetate, pH 8.50, was further purified on Amberlite CG-50 in 0.45 M ammonium acetate, pH 7.30. The four cardiotoxins were progressively eluted with increasing concentrations of the salt, CTX I at 0.45 M, CTX II and CTX III at 0.6 M, and CTX IV at 0.8 M. On the basis of amino acid analyses, these proteins are identical with cardiotoxins V''_1, V''_2, V''_4, and V''_3 obtained from the same venom by Louw (1974a), but with a different method. The sequence of these cardiotoxins is known (Louw, 1974b,c).

The cardiotoxins thus purified were generally contaminated (0.05–0.45% w/w) by a phospholipase A_2 type activity, present in large quantities in the venom and acting synergistically with the cardiotoxins (Condrea, 1974; Louw & Visser, 1978). A contamination level lower than 0.0003% has been obtained by chromatography of cardiotoxin samples on antiphospholipase A_2 γ-globulins–Sepharose CL-4B (Delori & Tessier, 1980). Only this last cardiotoxin preparation was used all through this study, and we never observed phospholipase activity.

CTX III (15 mg) was iodinated enzymatically with lactoperoxidase (Thorell & Johansson, 1971). A homogeneous fraction of diiodo-CTX III was obtained after chromatography on Amberlite CG-50 (specific radioactivity 14.8 mCi/mol).

† From the Laboratoire de Biochimie et Groupe 172 de l'INSERM, Faculté de Médecine, Secteur Nord, 13326 Marseille Cedex 3, France (P.B. and H.R.), and the Centre de Biochimie et de Biologie Moléculaire du CNRS, 13274 Marseille, France (G.P. and R.V.). *Received December 11, 1980.* This work is part of the thesis of P.B. obtained at l'Université d'Aix—Marseille II, Oct 9, 1980, and was supported in part by the DRET (79/469) and the CNRS (ERA 07 0617). The key concepts were presented during the CBB workshop on biochemical reactions at lipid–water interfaces held in Amsterdam, Dec 1–5, 1980.

0006-2960/81/0420-4915$01.25/0