Monoclonal Antibodies as Probes of Acetylcholine Receptor Structure. 1. Peptide Mapping†

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ABSTRACT: The isolated subunits of the acetylocholine receptor from *Torpedo californica* were digested with proteolytic enzymes, and the resulting polypeptide fragments were analyzed by gel electrophoresis. We have identified those fragments which contain carbohydrate and those from the α subunit which are labeled with the acetylcholine binding site specific reagent [4-(*N*-maleimido)benzyl]tri[^{3}H]methylammonium iodide. We have tested several monoclonal antibodies raised to the acetylcholine receptor from torpedo, some of which react with the denatured subunits [Tzartos, S. J., & Lindstrom, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755; Tzartos, S. J., & Lindstrom, J. M. (1981) in *Monclonal Antibodies in Endocrine Research* (Fellows, R., & Eisenbarth, G., Eds.) Raven Press (in press)]. The binding specificities of these antibodies to radioiodinated proteolytically generated fragments of the α subunit were determined by immunoprecipitation followed by gel electrophoresis. The antibodies tested fell into at least three main groups on the basis of their binding specificities. These antibodies were also tested for their capacity to bind to acetylcholine receptor in native membrane vesicles and to acetylcholine receptor solubilized in Triton X-100, sodium cholate, or sodium cholate supplemented with exogenous lipids. A monoclonal antibody raised to the denatured δ subunit, which cross-reacted with lower affinity with the γ subunit, was tested for its ability to select radioiodinated proteolytic fragments of these subunits. These molecules provide probes for many sites on the acetylcholine receptor with affinities and specificities comparable to α-neurotoxins.

Acetylcholine receptor (AcChR)[1] from the electric organ of *Torpedo californica* consists of four glycoprotein subunits in the ratio $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980). The apparent molecular weights of these subunits depend on the electrophoresis system and standards employed but are about 40 000, 50 000, 57 000, and 64 000, respectively (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1978; Chang & Bock, 1977; Lindstrom et al., 1978, 1979a; Froehner & Rafto, 1979). Within the 250 000 molecular weight monomer (Martinez-Carrion et al., 1975; Reynolds & Karlin, 1978) are acetylcholine binding sites and a cation specific channel (Anholt et al., 1980; Lindstrom et al., 1980a) which opens transiently when agonists are bound. All the subunits are closely associated in the membrane (Nathanson & Hall, 1980; Lindstrom et al., 1980c). α subunits contain part or all of the acetylcholine binding sites (Karlin & Cowburn, 1973; Damle & Karlin, 1978), but the functions of the other subunits are unknown.

Agonists, antagonists, snake venom toxins, and affinity labeling reagents have been used to probe the acetylcholine binding sites (Karlin & Cowburn, 1973; Sator et al., 1977; Damle & Karlin, 1978; Witzemann & Raftery, 1978; Witzemann et al., 1979; Hucho, 1979; Nathanson & Hall, 1980). Probes with specificity comparable to snake toxins have not been available for the cation channel and other parts of this complex macromolecule, although studies have been carried out using histrionicotoxin, local anesthetics, and phenylcyclidine (Briley & Changeux, 1977; Elliott et al., 1980; Albuquerque et al., 1980). A photoaffinity label of the local anesthetic binding site reacts only with the δ subunit (Waksman et al., 1980; Oswald et al., 1980; Saitoh et al., 1980). Still, most of the surface of this large macromolecule remains unexplored.

Antibodies to AcChR and its subunits are proving useful for studies of its structure and have been used to show the presence of subunits comparable to those observed in torpedo AcChR in AcChR from the electric organs of other species and in AcChR from mammalian muscle (Claudio & Raftery, 1977; Lindstrom et al., 1979b, 1980b).

Monoclonal antibodies (mAbs) have been prepared to AcChR by several groups (Gomez et al., 1979; Moshly-Rosen et al., 1979; Tzartos & Lindstrom, 1980, 1981; Lennon et al., 1980). mAbs have the potential to be excellent probes for AcChR because it should be possible to make mAbs (1) which are specific for many determinants on the surface of the AcChR molecule, (2) which react with affinities comparable to snake toxins, (3) which affect AcChR function by interfering with the acetylcholine binding site, regulation of the channel, or function of the channel itself, and (4) which interfere with AcChR metabolism and localization in vivo. We have begun to accumulate a library of mAbs to AcChR (Tzartos & Lindstrom, 1980, 1981) and now have more than 70 (S. J. Tzartos et al., unpublished results) with various species and subunit specificities.

For these mAbs to be most useful to us, we need to determine exactly where each binds to the AcChR molecule. Thus far, we have reported two mapping techniques (Tzartos & Lindstrom, 1980, 1981). One consists of immunoprecipitation of denatured ^{125}I-labeled subunits (Lindstrom et al., 1979b). In this way, we can map the subunit specificity of the mAbs to native AcChR whose binding specificity does not depend absolutely on the conformation of their antigenic determinant. This is about half the total mAbs. The other mapping technique uses mapped mAbs to localize unmapped mAbs by competitive binding to AcChR (Tzartos & Lindstrom, 1980, 1981). Here we report a method which is a variation of the

† From the Salk Institute for Biological Studies, San Diego, California 92122. *Received September 3, 1980.* This work was supported by grants from the National Institutes of Health (NS11323), the Muscular Dystrophy Association, and the Office of Naval Research. W.J.G. was supported by a fellowship from the Nobel Foundation and S.T. by a Muscular Dystrophy Association Fellowship.

[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; [^{125}I]αBGT, ^{125}I-labeled α-bungarotoxin; mAbs, monoclonal antibodies; [^{3}H]MBTA, [4-(*N*-maleimido)benzyl]tri[^{3}H]methylammonium iodide; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; torpedo, *Torpedo californica*.

0006-2960/81/0420-2173$01.25/0

immunoprecipitation method that permits mapping of mAbs to particular peptides from within a subunit. We have confined our attention primarily to α subunits because they are the best characterized and most of our mAbs are directed at this highly immunogenic subunit. This method has permitted some insights into localization of mAb binding sites on AcChR which can be related to studies of mAb binding to native AcChR described in the following paper of this issue (Conti-Tronconi et al., 1981). This method should become increasingly valuable as studies of mAb effects on AcChR function (Lindstrom et al., 1981) define important areas of the molecule.

AcChR subunits have been mapped both by resolving small tryptic peptides in two dimensions (Lindstrom et al., 1979a) and by separating larger proteolytic fragments in one dimension by electrophoresis on high-concentration polyacrylamide gels (Froehner & Rafto, 1979; Nathanson & Hall, 1979). The latter method has lower resolution but is much easier, so we have adapted it for our purposes. The large fragments obtained have the disadvantage of frequently overlapping, but many can be distinguished not only by size but also by the presence of carbohydrates and reaction with affinity-labeling reagents. In future studies, it should be possible to locate, for example, sites of phosphorylation (Vandlen et al., 1979; Gordon et al., 1979; Saitoh & Changeux, 1980) and local anesthetic binding (Saitoh et al., 1980) and more directly relate these peptides to the amino acid sequence of these subunits as it becomes known (Devillers-Thiery et al., 1979; Hunkapiller et al., 1979; Raftery et al., 1980).

Materials and Methods

Specimens of *T. californica* were obtained from Pacific Biomarine. AcChR was purified by affinity chromatography (Lindstrom et al., 1978). Subunits of AcChR were purified by preparative gel electrophoresis (Lindstrom et al., 1978, 1979a) and labeled with ^{125}I by using lactoperoxidase conjugated to agarose (David, 1971), which was a gift from Dr. Gary David. AcChR-rich membranes were purified by discontinuous and continuous density gradient centrifugation (Elliott et al., 1980).

Peptide mapping was performed essentially as described by Cleveland (Cleveland et al., 1977; Froehner & Rafto, 1979), and the resulting fragments were separated on $NaDodSO_4$–polyacrylamide slab gels by using the buffer system of Laemmli (1970). V8 protease (Miles) or papain (Sigma) was used to digest AcChR subunits. The molecular weight marker proteins used were phosphorylase B (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 000), and lysozyme (14 300) (Bio-Rad).

Peptide maps were stained for carbohydrate according to Glossman & Neville (1971) with the following modifications. After electrophoresis gels were washed in 40% methanol and 10% acetic acid for 90 min with three changes. Following oxidation, they were washed as above in 7% acetic acid. In this system, only glycosylated proteins gave detectable staining.

Soluble AcChR was affinity labeled with [^3H]MBTA (Karlin & Cowburn, 1973; Lindstrom et al., 1979b) and denatured in 125 mM Tris-HCl buffer, pH 6.8, containing 2% $NaDodSO_4$, 10% glycerol, and 1% β-mercaptoethanol ("sample buffer"). The labeled α subunit was purified by preparative gel electrophoresis.

Preparation of the mAbs used in this study was described previously (Tzartos & Lindstrom, 1980, 1981) and their numerical designations are the same. mAbs were reacted with subunit fragments as follows. Radioiodinated subunits (300 μL of $\sim 3 \times 10^{-7}$ M at 1.23×10^{18} cpm/mol) (Lindstrom et al., 1979b) in 0.1% $NaDodSO_4$ and 125 mM Tris-HCl buffer, pH 6.8, were digested with 150 μg of V8 protease for 30 min at room temperature. The reaction was stopped by making the solution 2% in $NaDodSO_4$ and 1 mM in PMSF and heating to 100 °C for 2 min to inactivate the protease. Detection of fragments bound to antibodies followed the method previously used with intact subunits (Lindstrom et al., 1979b). The radioactive fragments were diluted to 2.5×10^7 cpm/mL in 10 mM sodium phosphate buffer, pH 7.5, containing 0.5% Triton X-100, 100 mM sodium chloride, and 10 mM NaN_3 ("Triton buffer") (100 μL), and 1.5 μL of antiserum or 20 μL of mAb solution was added. The solutions were incubated overnight at 4 °C, and then 50 μL of goat antirat IgG antiserum, coupled to Sepharose Cl-4B (Pharmacia) (10 mg of protein/mL), was added and shaken for 2 h at 4 °C to bind the first antibody. The Sepharose–antibody complex was washed 5 times with 1 mL of Triton buffer and then twice with 1 mL of distilled water. Sample buffer (70 μL), without β-mercaptoethanol, was added and left for 30 min to dissociate the subunit fragments together with the first antibody. Samples of the supernatant were counted, and, where possible, equal amounts of radioactive protein per track were electrophoresed on 15% $NaDodSO_4$–polyacrylamide gels. Concentrations of antiserum or mAb were chosen, based on their titer, to provide a 5-fold antigen excess. In the case of cross-reacting antisubunit sera, the same volume of heterologous antiserum was added as the homologous antiserum. Also, in this instance, the gel loading was such that the maximum volume (50 μL) of fragments eluted from the goat antirat IgG Sepharose was applied for the cross-reacting mAb 7, and the other tracks were loaded with an equal amount of radioactivity where possible. After electrophoresis the gels were fixed, washed, and dried. The dried gels were autoradiographed by using preflashed Royal X-Omat film and an intensifying screen (Swanstrom & Shank, 1978; Du Pont, Cronex).

AcChR-rich membrane vesicles were trace labeled with 0.1 mol equiv of [^{125}I]αBGT (specific activity 2×10^{17} cpm/mol). The labeled vesicles were diluted to 2×10^{-11} mol of AcChR/mL in either 10 mM sodium phosphate buffer, pH 7.5, containing 145 mM sucrose and 5 mM NaN_3 ("flux buffer") or one of the following: 1% Triton X-100 (Sigma), 1% sodium cholate (Interchem, Matheson), or 1% sodium cholate containing 2.5 mg/mL soybean lipids (L-α-phosphatidylcholine; Sigma) in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 10 mM NaN_3. Antiserum was added in duplicate to 1-mL samples of intact (in flux buffer) or solubilized (in detergent) vesicles in 2-fold molar excess based in titer (Tzartos & Lindstrom, 1980, 1981) and left for 4–16 h. The intact vesicles were centrifuged at 8000 rpm for 15 min in an Eppendorf microcentrifuge. The supernatant, containing unbound antibodies, was aspirated and the pellet of intact labeled vesicles and bound antibody dissolved in 1 mL of Triton buffer. Goat antirat immunoglobulin and 5 μL of normal serum as carrier were added to both the solubilized pellet and the "detergent solubilized" vesicles, and the tubes were left for 4 h at 4 °C. The precipitate was pelleted and washed once with 1 mL of Triton buffer, and its radioactive content was determined in a γ counter.

Results

The subunits of AcChR were separated (Lindstrom et al., 1979a) and subjected to peptide mapping in $NaDodSO_4$–polyacrylamide gels by using the method of Cleveland (Cleveland et al., 1977). V8 protease and papain gave patterns of digestion of the α, β, γ, and δ subunits similar to those obtained by other workers (Froehner & Rafto, 1979) (Figure

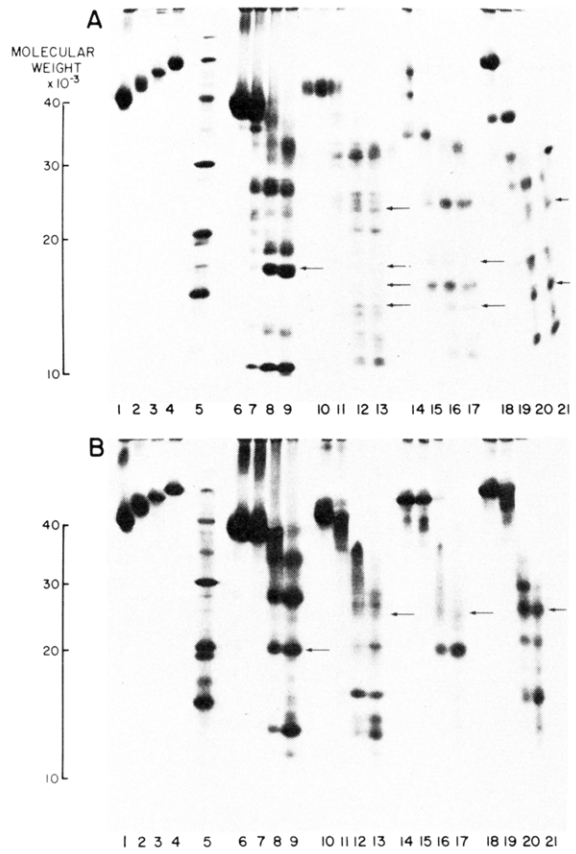

FIGURE 1: Peptide mapping on $NaDodSO_4$–polyacrylamide gels of the subunits of AcChR. (A) Peptide map using V8 protease. (1–4) 5 μg of α, β, γ, and δ subunits, (5) molecular weight marker proteins, (6–9) α, (10–13) β, (14–17) γ, and (18–21) δ digested with 0.0005, 0.005, 0.05, and 0.25 μg of V8 protease, respectively. (B) Peptide map using papain. (1–4) 5 μg of α, β, γ, and δ subunits, (5) molecular weight markers, (6–9) α, (10–13) β, (14–17) γ, and (18–21) δ digested with 0.0001, 0.001, 0.01 and 0.1 μg of papain, respectively. Arrows indicate those bands which were stained with periodate–Schiff reagent to detect carbohydrate.

1A,B). Characteristic peptides were formed with increasing amounts of protease, but above a certain ratio, the pattern remained constant over a fairly wide range of protease concentration (see, for instance, Figure 1A, tracks 8 and 9). There were few fragments of the same molecular weight present in different subunit digestion patterns. The enzymes α-chymotrypsin, elastase, proteinase K, and thermolysin generated larger numbers of overlapping fragments (not shown) and were not used further in this work.

Peptide maps were stained with periodate–Schiff reagent (Glossman & Neville, 1971) to detect those fragment(s) which contain carbohydrate. Only one fragment of the α subunit contained a significant amount of carbohydrate, whether digested with V8 protease (Figure 1A, track 9, apparent molecular weight 17 000) or papain (Figure 1B, track 9, apparent molecular weight 21 000). These fragments were also strongly labeled with ^{125}I-labeled concanavalin A, confirming the presence of carbohydrate, but by this method some other bands were weakly labeled (data not shown). Digestion of β, γ, or δ subunits with papain gave in each instance only single fragments which contained significant amounts of carbohydrate. The molecular weights of these fragments were the same (molecular weight 25 000). Digestion of the same group of subunits with V8 protease gave a number of rather weakly staining bands. Studies of other membrane proteins (Marchesi et al., 1976) suggest that carbohydrate-containing portions are located on the extracellular surface.

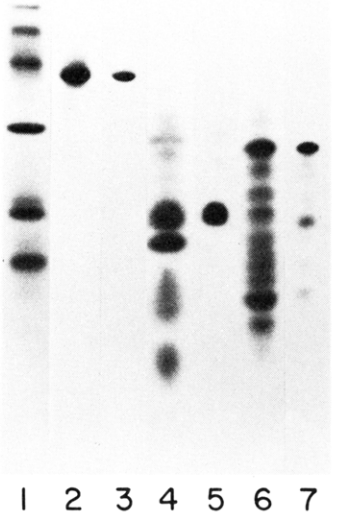

FIGURE 2: Peptide mapping of [^{3}H]MBTA-labeled AcChR. (1) Molecular weight marker proteins, (2) [^{3}H]MBTA-labeled α subunit, (4) [^{3}H]MBTA α subunit (10 μg) digested with 1.0 μg of V8 protease, (6) [^{3}H]MBTA α subunit (10 μg) digested with 0.4 μg of papain, (3, 5, 7) autoradiograph of 2, 4, and 6, respectively.

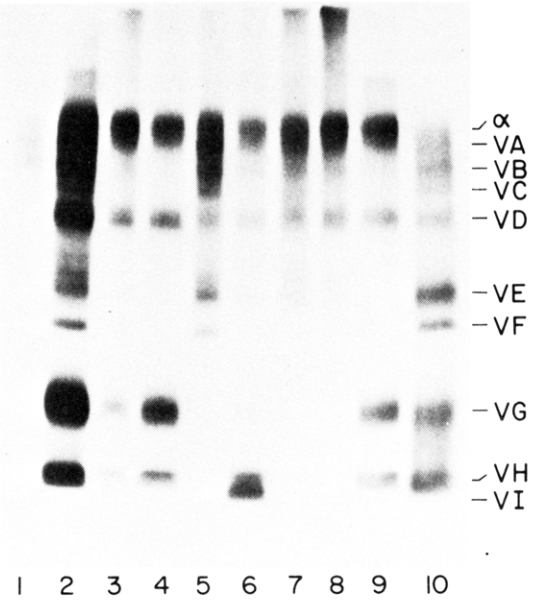

FIGURE 3: Immunoprecipitation of fragments of the ^{125}I-labeled α subunit digested with V8 protease. (1) Normal rat serum, (2) antiserum to denatured α, (3) mAb 3, (4) mAb 5, (5) mAb 6, (6) mAb 8, (7) mAb 10, (8) mAb 13, (9) mAb 19, (10) ^{125}I-labeled α digested with V8 protease.

Native AcChR was labeled with the acetylcholine binding site specific reagent [^{3}H]MBTA (Weill et al., 1974), and the subunits were separated by preparative gel electrophoresis. Only the α subunit was labeled. Digestion with large amounts of V8 protease (Figure 2) gave only a single labeled polypeptide fragment whereas papain gave two. Digestion with low amounts of V8 protease generated a higher proportion of high molecular weight fragments, several of which were labeled (data not shown). At least part of the peptides containing the [^{3}H]MBTA would be expected to be exposed on the extracellular surface of the AcChR.

The isolated α subunit was labeled with ^{125}I (Lindstrom et al., 1979a), the peptide was mapped with V8 protease and papain, and the gels were autoradiographed. Both proteases gave a fragment pattern resembling that obtained by Coomassie blue staining (cf. Figure 1A, track 9, and Figure 3,

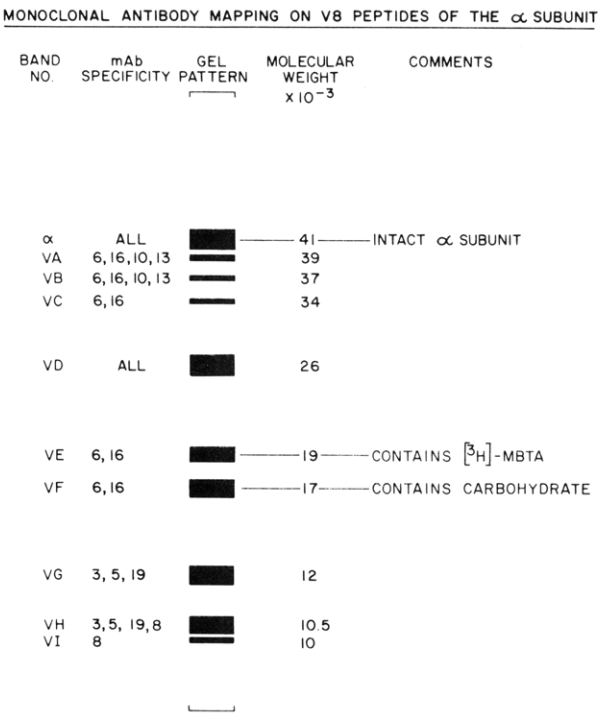

FIGURE 4: Summary of the molecular weights, carbohydrate content, MBTA reaction, and mAb binding specificities of fragments of the α subunit produced by V8 protease digestion.

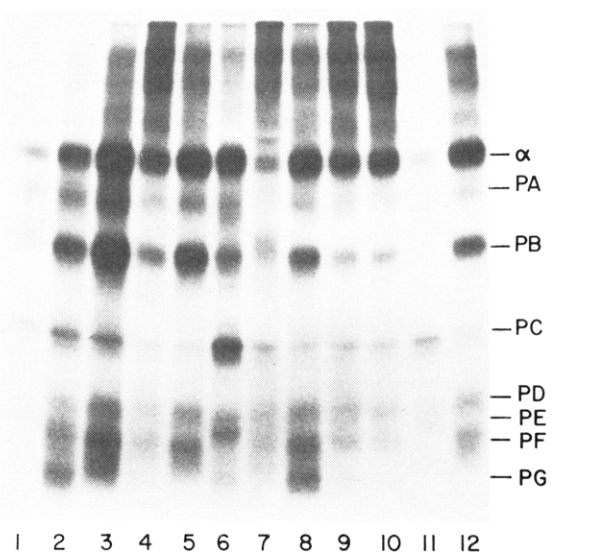

FIGURE 5: Immunoprecipitation of fragments of ^{125}I-labeled α subunit digested with papain. (1) Normal rat serum, (2) ^{125}I-labeled α subunit digested with papain, (3) antiserum to denatured α, (4) mAb 3, (5) mAb 5, (6) mAb 6, (7) mAb 7, (8) mAb 8, (9) mAb 10, (10) mAb 13, (11) mAb 16, (12) mAb 19.

track 10; cf. Figure 1B, track 9, and Figure 5, track 2), except that the intensities of the bands were different. The peptide fragments were assigned identifying letters for the subunit and protease used, in order of decreasing molecular weight (Figures 3 and 5). Very high enzyme/substrate ratios were required to generate patterns of fragments resembling those produced by unlabeled material. This may be due to the relatively low concentration of radioactive protein in the digestion mixture (0.3 μg/mL) or to steric interference of the enzyme action by those residues substituted with bulky iodine atoms. The latter is unlikely since V8 protease is specific for glutamate and aspartate residues (Houmard & Drapeau, 1972), neither of which is iodinated.

The proteases were inactivated by heating at 100 °C in 2% $NaDodSO_4$ for 2 min, and the solution was diluted to approximately 5×10^{-9} mol of subunit/L in 0.1% $NaDodSO_4$ and 0.5% Triton. Antisubunit serum or mAb was added, followed by antiimmunoglobulin coupled to Sepharose CL-4B, and the immune complex was washed thoroughly. Those fragments of the α subunit which were bound were detected by dissociating the complex in $NaDodSO_4$ followed by gel electrophoresis and autoradiography (Figures 3 and 5).

There was negligible precipitation of V8 protease produced fragments with nonimmune rat serum (Figure 3, track 1). Antiserum raised to $NaDodSO_4$-denatured intact α subunits precipitated all the fragments (Figure 3, track 2) present in the total digestion mixture (Figure 3, track 10). This shows that there are many antigenic determinants spread throughout the denatured chains. It is apparent that bands VE and VF, containing the [^{3}H]MBTA binding site and carbohydrate, respectively, were precipitated to a lesser extent than the other fragments in comparison to their relative abundance in the total digest.

The mAbs tested form at least three groups with regard to their reaction with the V8 protease fragments of the α subunit. These groups are consistent with groups of mAbs previously described (Tzartos & Lindstrom, 1980, 1981) which compete for binding to AcChR. All the mAbs reacted rather well with the trace of intact α subunit present after digestion. The intensity of this band in the autoradiograph is, however, amplified by its higher radioactivity per mole than that of the smaller fragments. mAbs 6 (Figure 3, track 5) and 16 (not shown) bind to fragments VE and VF (and bands VA, VB, VC, and VD and intact subunit) whereas mAbs 3, 5, and 19 bind the fragment groups VD, VG, and VH (Figure 3, tracks 3, 4, and 9, respectively) (see Figure 4). The fragments precipitated by mAb 10 and 13 include VD and traces of VE. mAb 8 reacts with fragments VD and VH (as do 3, 5, and 19) but not with fragment VG and uniquely brings down fragment VI. Thus, mAb 8 is in a different category than mAbs 3, 5, and 19 and reacts with a determinant present on fragments VH, VI, and VD but not present on fragment VG. (These results are summarized in Figure 4.)

The reaction of mAbs with papain-generated fragments of the α subunit is shown in Figure 5. There is negligible binding by nonimmune rat serum (Figure 5, track 1), and anti-α-subunit antiserum (Figure 5, track 3) binds all the fragments present in the total digestion mixture (Figure 5, track 2). The mAbs again fall into the same type of groups as may be defined by their reactions with V8 fragments of the α subunit or by binding competition to the native AcChR (Tzartos & Lindstrom, 1980, 1981). mAbs 3, 5, and 19 (Figure 5, tracks 4, 5, and 12) each gave essentially the same pattern, binding fragments PA (weakly), PB (strongly), and PD and PF. mAbs 6 and 16 (Figure 5, tracks 6 and 11) gave a distinctly different pattern, reacting with fragment PC (strongly) and PE and relatively less with PB and PA. mAb 16 did not bring down much radioactive material, reflecting its low titer against the denatured α subunit (Tzartos & Lindstrom, 1980, 1981). mAbs 10 and 13 react weakly with essentially all the fragments except PG. Interestingly, mAb 8 (Figure 5, track 8) again binds fragments PA (weakly), PB (strongly), and PD and PF as do mAbs 3, 5, and 19 but additionally reacts strongly and uniquely with fragment PG (summarized in Figure 6).

All or most of the fragments of the α subunit digested with either enzyme contain some overlapping regions since the sum of their molecular weights greatly exceeds that of the intact α subunit. mAbs 6 and 16 react with V8 protease fragments VE and VF, which contain the [^{3}H]MBTA binding site or

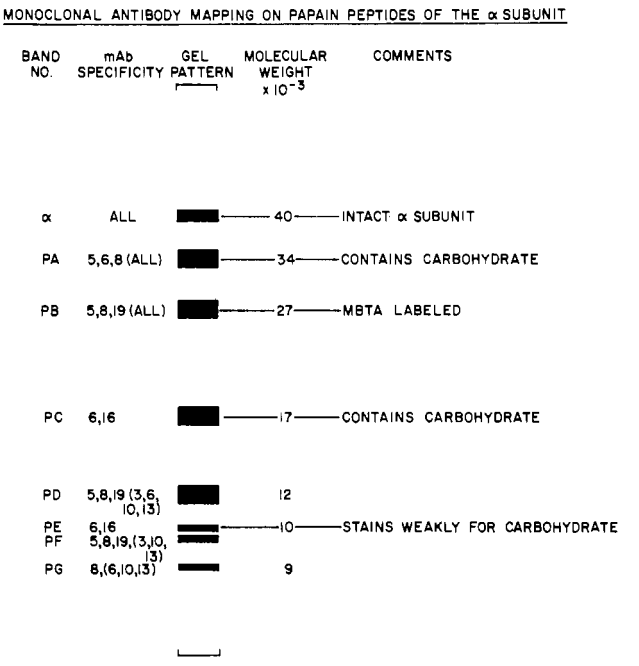

FIGURE 6: Summary of the molecular weights, carbohydrate content, MBTA reaction, and mAb binding specificities of fragments of the α subunit produced by papain digestion. The mAbs shown in parentheses gave a weak precipitation of the fragment indicated.

carbohydrate, and with fragments PC and PE, both of which contain carbohydrate. These sequences must be at least partially exposed to the extracellular aqueous environment. Some part of the α subunit is, however, known to be buried within the cell membrane (Tarrab-Hazdai et al., 1980). Part or all of the other fragments may be exposed to the aqueous extracellular compartment, interact with other subunits, be folded into the hydrophobic subunit core, interact with membrane lipids, or be exposed to the cytoplasmic compartment.

In order to determine which antigenic determinants are exposed on the extracellular oriented surface of the AcChR, we purified AcChR-rich vesicles from *Torpedo californica* electroplax (Elliott et al., 1980) and tested the extent of binding of some mAbs. AcChR in vesicles was trace labeled with [^{125}I]αBGT. The problem of the orientation of the AcChR was avoided since only AcChR in "correctly" oriented vesicles (>95%) would be labeled and thus appear in the antibody binding assay. Samples of labeled vesicles were diluted into different detergent solutions to solubilize the vesicles and the extent of mAb binding was determined. Vesicles were diluted into 10% sodium cholate and left for 5 min to completely solubilize the membranes. This solution was then diluted to 1% sodium cholate and the antiserum or mAb added. Samples were diluted into 4% sodium cholate and 10 mg/mL soybean lipid and then subsequently to 1% sodium cholate and 2.5 mg/mL lipid in order to completely solubilize the native membranes. Vesicles were also diluted directly into 1% Triton X-100, which was sufficient to solubilize the membranes. Trace-labeled intact and detergent-solubilized vesicles were allowed to react with antisera. The solutions containing native vesicles were centrifuged to pellet the vesicles, and the unbound antibodies were removed. The pellet containing AcChR and bound antibodfies was resuspended in Triton buffer. Goat antirat IgG antiserum was added to all the samples, and the resulting precipitate was washed and its radioactivity determined. Normal rat serum and rat anti-native-AcChR antiserum were added to both vesicles and detergent-solubilized AcChR to act as 0% and 100% reaction standards. The reaction of the mAbs, as a percentage of the total reaction (with rat anti-native-AcChR antiserum), minus the small amount of nonspecific reaction (with normal rat serum), is given in Table I. Since a 2-fold excess of mAb titer over AcChR concentration was added, generally a near-maximal precipitation was observed by mAbs with the Triton-solubilized AcChR. mAbs 3 and 13, however, only precipitated 60–70% of the maximum and mAb 9 only approximately 30% (Table I). Increasing the mAb titer/AcChR ratio of these three antibodies to 10 increased their binding significantly, indicating that their low extent of precipitation was due to their low binding affinity to the native AcChR [see Tzartos & Lindstrom (1981) for direct measurements of their relatively low K_D's ((4–40) × 10^{-9} M)]. The extent of reaction of mAbs with AcChR in intact native vesicles was more varied. mAb 6 was predicted to react well with AcChR in vesicles since part or all of the peptides it recognized appeared to be exposed to the aqueous environment. It bound very well to membrane-bound and soluble AcChR (Table I). Thus as expected, the detergent type or integration of AcChR into membrane did not affect the binding to any greater extent. mAbs 3, 5, and 19, however, all reacted well with solubilized AcChR but bound much less to the AcChR in the native vesicles (Table I). mAb 8 seemed to bind well to AcChR in Triton, moderately well in cholate, less well in cholate/lipid, and essentially not at all in native vesicles (2 ± 1.0%). mAbs 10 and 13 bound fairly well to AcChR solubilized in Triton. However, they bound to quite different extends to AcChR in native vesicles (mAb 10, 12 ± 2.5%; mAb 13, 67 ± 3.1%).

Table I: Monoclonal Antibody Binding to AcChR in Solution and in the Membrane

mAb[a]	1% Triton (mean ± SE)	1% cholate (mean ± SE)	1% cholate + 2.5 mg/mL lipid (mean ± SE)	native vesicles (mean ± SE)
1	92 ± 2.2	91 ± 1.6	85 ± 2.1	77 ± 3.5
2	92 ± 2.9	79 ± 4.2	75 ± 4.3	75 ± 4.5
3	68 ± 4.9	62 ± 3.5	64 ± 6.0	9 ± 0.4
4	79 ± 5.5	88 ± 1.5	82 ± 3.5	78 ± 3.0
5	80 ± 5.0	80 ± 2.6	87 ± 3.7	28 ± 1.9
6	94 ± 1.6	80 ± 3.8	67 ± 4.0	77 ± 5.0
7	89 ± 2.3	77 ± 1.9	84 ± 1.7	42 ± 2.3
8	90 ± 2.6	62 ± 5.6	25 ± 2.1	2 ± 1.0
9	32 ± 2.0			25 ± 3.0
10	95			12 ± 2.5
11	81 ± 2.7	76 ± 4.9	77 ± 3.3	9 ± 1
12	80 ± 2.8	86 ± 3.1	85 ± 2.9	83 ± 4.2
13	63			67 ± 3.1
14	84 ± 2.5	77 ± 0.6	71 ± 4.6	65 ± 4.0
16	98 ± 1	51 ± 5.0	62 ± 3.5	29 ± 2.9
17	88 ± 1.5	90 ± 3.7	86 ± 1.8	87 ± 2.6
19	88 ± 2.9	85 ± 3.2	94 ± 3.2	41 ± 6.6

[a] Antibody was added at double the AcChR concentration based on AcChR titer.

In our initial studies, we observed that mAb 7, which was obtained from an animal immunized with the δ subunit of AcChR from *Torpedo californica*, cross-reacted at lower affinity with the γ subunit (Tzartos & Lindstrom, 1980). We also observed that it cross-reacted with the corresponding subunits of the AcChR from *Electrophorus electricus* electric organ (Lindstrom et al., 1980b). Raftery and co-workers (1980) have subsequently demonstrated that there is significant amino acid sequence homology between all the subunits of torpedo AcChR in their N-terminal regions, which helps to explain these observations. We investigated whether the sites at which mAb 7 reacts on the γ and δ subunits are located on corresponding peptide fragments of the two subunits.

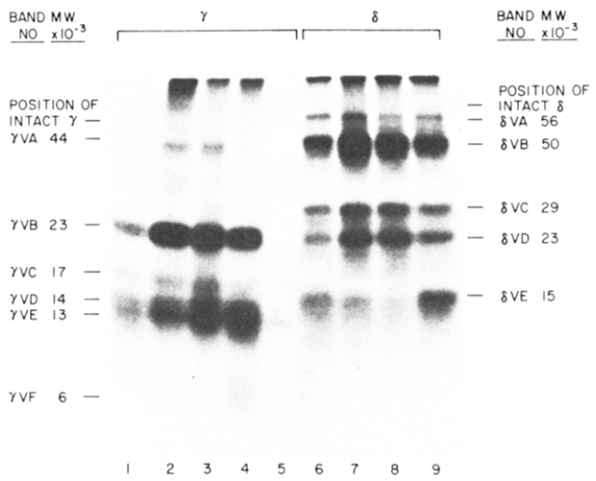

FIGURE 7: Immunoprecipitation of fragments of the ^{125}I-labeled γ and δ subunits digested with V8 protease. (1) ^{125}I-Labeled γ subunit digested with V8 protease, (3–5) ^{125}I-labeled γ subunit digested with V8 protease [fragments bound by (2) antiserum to γ, (3) antiserum to δ, (4) mAb 7, (5) mAb 6], (6) ^{125}I-labeled δ subunit digested with V8 protease, (6–9) ^{125}I-labeled δ subunit digested with V8 protease [fragments bound by (7) antiserum to δ, (8) antiserum to γ, and (9) mAb 7].

Peptide maps of iodinated γ and δ subunits were obtained by using V8 protease (Figure 7, tracks 1 and 6, respectively), which resembled the patterns obtained with Coomassie blue stained material. Antiserum to the γ subunit bound to all the fragments of the γ subunit (Figure 7, track 2), but there was little nonspecific binding by an irrelevant antibody, mAb 6 (Figure 7, track 5). Antiserum to the δ subunit also bound a small amount of each of the γ subunit fragments (Figure 7, track 3) (equal quantities of radioactive material were applied to each track where possible, amplifying the intensity of the material bound by the heterologous antisubunit antisera). mAb 7 bound to fragments γVB, γVD, γVE, and γVF. The latter band appears rather broad and diffuse, and its molecular weight was estimated to be approximately 6000. It is, however, clearly present in track 4 but is not detectable in the pattern of fragments brought down by the subunit antisera. The δ subunit of torpedo AcChR was also digested with V8 protease, and the binding specificities of the same group of antisera and mAbs were investigated. Antiserum to the δ subunit (Figure 7, track 7) binds to all of the fragments of the δ subunit (Figure 7, track 6) whereas irrelevant mAbs, such as mAb 6, do not bind significantly (not shown). Antiserum to the γ subunit binds to all the fragments precipitated by the homologous antisubunit serum, but it does so less efficiently. mAb 7 reacts with all the fragments of δ, but particularly strongly with fragment δVE.

Discussion

The development of mAbs which cross-react between subunits of torpedo AcChR (Tzartos & Lindstrom, 1980, 1981) and the recent report of extensive sequence homology between the N-terminal 57 amino acid residues of all the subunits (Raftery et al., 1980) strongly indicate that the structures of the four types of subunit present in an AcChR monomer are significantly related. There are, however, sufficient differences in their sizes and sequences that specific subunit antisera can be raised (Claudio & Raftery, 1977; Lindstrom et al., 1978, 1980b) and unique peptide maps derived (Froehner & Rafto, 1979). Peptide mapping in $NaDodSO_4$ gels (Cleveland et al., 1977) can detect only rather large protein fragments (>5000 molecular weight), which would indicate homologies between proteins only if extensive regions of identical or very similar sequences existed (Calvert & Gratzer, 1978). The discovery that the N-terminal regions of the γ and δ subunits possess 50% amino acid homology provides an explanation for the differences observed in the peptide maps and, at the same time, for the existence of cross-reacting mAbs.

In this work, we digested the purified subunits of torpedo AcChR to generate peptide maps. Although the rather large peptides obtained had the disadvantage that many were overlapping, many could be distinguished by molecular weight, carbohydrate content, MBTA reaction, and reaction with mAbs. Peptide maps were stained by the periodate–Schiff method to detect those fragments that contained carbohydrate. Isolated subunits digested with papain, in each case, gave a single positively staining band (Figure 1B, tracks 9, 13, 17, and 21). The molecular weight of the fragments derived from the β, γ, and δ subunits was the same (25 000), but it is not clear whether this represents a significant structural relationship or is merely coincidental. V8 protease digested subunits gave a more complicated picture. Only one fragment of the α subunit was stained, but the β, γ, and δ subunit patterns showed many weakly staining bands, only the major ones being indicated in Figure 1A.

Native AcChR was labeled by the affinity alkylating reagent [^{3}H]MBTA which, under these conditions, reacts with a sulfhydryl group on one of the two α subunits located at, or close to, the AcCh binding site (Damle & Karlin, 1978). A mixture of the labeled and unlabeled α subunits digested with a high amount of V8 protease gave a single labeled fragment, different in molecular weight from that which contained carbohydrate. These experiments do not indicate whether, in the native AcChR, the AcCh binding site and the carbohydrate are juxtaposed by tertiary folding. It is clear, however, that the polypeptide fragments on which they lie must be at least partially exposed to the extracellular aqueous environment.

The α subunit of AcChR, isolated by preparative gel electrophoresis, was radioiodinated and then digested with V8 protease or papain. The fragment patterns generated closely resembled those observed by Coomassie blue staining of digested, unlabeled α subunit, although the intensity of staining and the extent of radiolabeling differed. Antisera raised to the denatured α subunit reacted with all the fragments but, in the case of the V8 proteolyzed α subunit, rather poorly with the fragments VE and VF which contain the AcCh binding site or carbohydrate. mAbs which react with $NaDodSO_4$-denatured α subunits (Tzartos & Lindstrom, 1980, 1981) were tested for their ability to select fragments, containing the antigenic determinant against which they are directed, from the digestion products of the α subunit. The mAbs employed fell into three groups on the basis of their selection of fragments (summarized in Figures 4 and 6). mAbs 3, 5, and 19 reacted with the same fragment specificities, whether against the V8 or papain-digested α subunit. Clearly their determinant(s) must be very close, if not identical. It is equally clear that, unless they are directed against a repeated determinant, the fragments to which they bind must contain overlapping sequences. mAb 8 binds to some of the same fragments as do mAbs 3, 5, and 19, but not to all, and mAb 8 binds to an additional relatively small fragment PG (molecular weight 9000) from papain-digested α subunit and VI (molecular weight 10 000) from V8 digested α. mAbs 3 and 5 have been found to compete with each other, but not with mAb 8, when binding to native AcChR. This shows that in the native molecule the determinant for mAbs 3 and 5 is located apart from that for mAb 8 (Tzartos & Lindstrom, 1980, 1981; Conti-Tronconi et al., 1981). mAbs 6 and 16 bring down the

same fragment group, which is distinct from the fragment groups bound by mAbs 3, 5, and 19 or mAb 8. Therefore, mAbs 6 and 16 define a third distinct antigenic determinant on α. mAbs 6 and 16 do not compete with either mAbs 3 and 5 or mAb 8 in binding to native AcChR (Tzartos & Lindstrom, 1980, 1981; Conti-Tronconi et al., 1981). Neither mAb 6 nor 16 reacts well with denatured α or its proteolytic fragments; indeed, mAb 16 hardly reacts at all. The highly immunogenic determinant on the native α subunit against which mAb 6 is directed [see Tzartos & Lindstrom (1980, 1981)] is therefore almost entirely destroyed as a result of denaturation and $NaDodSO_4$ binding.

In order to determine the orientation of the fragments of the α subunit in native AcChR, we prepared AcChR-rich membrane vesicles and determined the extent of mAb binding in comparison to that observed with solubilized AcChR. It is important to note that the two systems differ by necessity. Whereas mAbs added to solubilized AcChR remained bound or free in solution throughout the assay, unbound mAbs added to the vesicles were removed after the centrifugation step. Thus, mAbs with low affinities for AcChR in membrane may partition themselves between those bound to AcChR and those free in solution during precipitation with the second antibody. This effect, however, should not be significant with those mAbs which have a high affinity for the AcChR. Native vesicles were solubilized in Triton X-100, sodium cholate, or sodium cholate supplemented with exogenous lipids. Solubilization in detergents alone irreversibly denatures the AcChR cation channel, but the channel is protected by cholate–lipid mixtures.

All the mAbs tested bound well to AcChR in Triton or cholate, with the exception of mAbs 3, 9, and 13 which bound to a lesser extent due to their low affinities. mAbs 8 and 16 bound less well to AcChR in cholate than in Triton. The most likely explanations are that AcChR binds cholate to a greater extent than Triton, causing steric hindrance of antibody binding, or that AcChR exists in different conformational states in the two detergents. There were no significant differences in mAb binding between AcChR solubilized in cholate or AcChR solubilized in cholate containing lipid, with the single exception of mAb 8, which bound much less in the latter system. The determinant to which mAb 8 binds may be involved in a conformational change accompanying solubilization or may be sterically inhibited from binding the mAb by bound lipid. The latter hypothesis appears more likely since mAb 8 does not bind to AcChR in native membrane. Other mAbs such as 3, 5, 10, 11, and 19 bind poorly to AcChR in native vesicles but bind relatively well to AcChR solubilized in any of the detergent systems. This group is discouraged from binding either by the close packing of AcChR in native membranes or simply due to steric hindrance by the membrane itself. There is thus a unique progression of decreased binding by mAb 8 from Triton > cholate > cholate/lipid > native vesicles. mAb 8 is also unique among those antibodies tested with regard to its peptide fragment binding specificity. An appealing hypothesis is that it binds to a region of the α subunit exposed to the lumen of the vesicles. This appears unlikely, however, since it binds to approximately 10% of labeled (and therefore correctly oriented) AcChR in reconstituted vesicles (unpublished experiments). The absence of binding to native vesicles and poor binding to reconstituted vesicles and to AcChR in cholate/lipid mixture may be a result of steric hindrance due to the close packing of lipids around the AcChR.

mAb 6, which reacts with fragments VE and VF of the α subunit, binds well to both membrane-bound and solubilized AcChR. This finding is consistent with experiments which indicated that all or part of these fragments are oriented toward the extracellular compartment since they contain carbohydrate and the [^{3}H]MBTA binding site. mAb 16, however, which also reacts with these fragments, binds less well to membrane-bound AcChR. Its binding affinity for solubilized AcChR is 30-fold lower than that of mAb 6 (Tzartos & Lindstrom, 1981). The affinity of both mAbs may be lower for membrane-bound AcChR, but it may be evident only with mAb 16.

Those mAbs obtained from animals immunized with AcChR in vesicles (1, 2, 4, and 6) all react well with AcChR in vesicles and with solubilized AcChR. mAbs 8–11, however, which were obtained from animals immunized with denatured AcChR subunits, all react poorly with AcChR in vesicles, but much more strongly with solubilized AcChR. It seems, therefore, that there is a tendency in the latter group to recognize parts of the molecule which are available for reaction only in the solubilized or denatured AcChR.

mAbs have been developed which cross-react between α and β, and γ and δ subunits. Indeed, no mAb has so far been obtained that reacts exclusively with the γ subunit (Tzartos & Lindstrom, 1980, 1981; S. J. Tzartos and J. M. Lindstrom, unpublished experiments). Antiserum raised against the δ subunit will bind all the fragments of the γ subunit, and vice versa, but in each instance only approximately 10% as efficiently as the homologous antiserum. This indicates either that the immunizing antigen was contaminated with other subunits or that the subunits contain similar determinants. The latter has clearly been demonstrated by partial sequence analysis (Raftery et al., 1980). Since mAbs which cross-react between subunits have frequently been obtained, it would be predicted that antisera to subunits would contain populations of cross-reacting antibodies with varying affinities for their determinants. The determinant(s) to which mAb 7 binds does (do) not appear to be carbohydrate since only two V8 protease produced fragments of either subunit were stained with periodate–Schiff reagent. However, the binding is specific since mAb 7 showed selective binding of γ-subunit fragments. The fact that essentially all the δ-subunit fragments were bound by mAb 7 is surprising. Either there is nonspecific binding to a number of similar, perhaps charge-type determinants (Atassi, 1977), or all the δ-subunit fragments are overlapping, which is unlikely since not all stain for carbohydrate, or the antibody binding is specific but to a repeated sequence determinant. The use of chemical cleavage to provide unambiguously nonoverlapping fragments is in progress and should provide answers to these questions. Clearly, however, the single cross-reacting mAb studied here cannot indicate the extent of sequence homology between cross-reacting subunits. This laboratory has, however, produced 70 mAbs, some of which cross-react with pairs of subunits which may allow this question to be rapidly answered.

The object of this work has been to probe the structure of the AcChR molecule by using the technique of peptide mapping to produce large fragments of each isolated subunit. The combination of this method with the use of affinity-labeling, specific-staining techniques and the binding of mAbs directed against highly specified structural components has allowed resolution of structural features with a higher definition than has been available previously. mAbs which inhibit carbamoylcholine-induced $^{22}Na^+$ influx into AcChR-containing vesicles without simply inhibiting toxin binding have been obtained in this laboratory (Lindstrom et al., 1981). The subunit(s) and the regions within subunit(s) to which they bind may be determined by using the methods developed here.

Acknowledgments

We thank Brett Einarson for diligent and thoughtful technical assistance, John Cooper, Vernita Hudson, Debbie Skelly, and Diane Rand for technical assistance, and Jan Littrell for typing the manuscript.

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