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The Antigenic Structure of the Acetylcholine Receptor From Torpedo californica

W.J. Gullick and J.M. Lindstrom

The Salk Institute for Biological Studies, San Diego, California 92138

The immunological structure of the acetylcholine receptor (AChR) from the electric organ of Torpedo californica was studied using a large number of monoclonal antibodies which were initially selected for their abilities to bind to intact AChRs. The monoclonal antibodies were tested for their ability to bind to denatured AChR subunits labeled with ^{125}I. Antibodies derived from rats immunized with individual denatured subunits or a mixture of subunits of Torpedo AChR reacted well in the assay. A much smaller proportion of antibodies derived from rats immunized with native Torpedo AChR or native AChR from Electrophorus electricus electric organ, bovine muscle, or human muscle reacted with denatured subunits of Torpedo AChR. Many monoclonal antibodies reacted with more than one subunit, but they always reacted best with the subunit used for immunization.

Those monoclonal antibodies that bound to intact subunits were mapped more precisely by their ability to bind characteristic fragments of each subunit generated by proteolysis with Staphylococcal V8 protease. These fragments were analyzed by SDS polyacrylamide gel electrophoresis, and monoclonal antibodies that precipitated the same fragment pattern were placed in groups. By this method, we define a minimum of 28 determinants on Torpedo AChR.

Key words: peptide mapping, monoclonal antibodies, acetylcholine receptor

Acetylcholine receptor (AChR) is a multisubunit transmembrane glycoprotein in the postsynaptic membrane of vertebrate neuromuscular junctions and is present in high concentrations in the electric organs of some fish [1, 2]. The function of the AChR is to respond to binding acetylcholine released by the nerve terminal by opening a channel through the postsynaptic membrane through which sodium and potassium flow passively to create a local depolarization which triggers an action potential in the muscle that leads to muscle contraction.

The AChR from the electric organ of Torpedo californica is composed of four types of subunits, α (MW 40,000), β (MW 50,000), γ (MW 57,000), and δ (MW

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64,000) arranged in a 250,000-MW complex with the subunit-stoichiometry $\alpha_2\beta\gamma\delta$ [3–5]. Acetylcholine binding sites are located on the two α subunits [1, 2] and the cation channel whose opening they regulate is part of this $\alpha_2\beta\gamma\delta$ structure [6,7], but the structure of the channel and the functions of the β, γ, and δ subunits are unknown. There are many probes for the ACh binding sites (snake venom toxins [8], affinity labeling reagents [2], agonists, and antagonists), but until the development of monoclonal antibodies (mAbs), specific probes for most of the surface of this large and complex molecule were unavailable. We have now generated a large library of mAbs to the AChR [9–12].

One of the purposes of this library is to define structures on the AChR molecule [9, 10, 13, 14] and, by using mAbs as probes, to determine their function [15]. Because many of these mAbs cross-react between species, the mAbs should also be able to function as templates for comparing AChRs from various tissues (eg, electric organ, muscle, and brain) and various species (eg, fish, cows, and humans) by taking advantage of the ability to map mAb specificities precisely on the biochemically accessible AChRs of Torpedo and then using these mAbs to identify homologous structures on AChRs available in small quantities such as those from human muscle [15, 16].

Myasthenia gravis is an autoimmune disease in which antibodies to AChRs cause muscular weakness by impairing neuromuscular transmission [17, 18]. Experimental autoimmune myasthenia gravis is produced by immunizing animals with purified AChRs [19]. Another use to which we are putting these mAbs is as model autoantibodies to study the role of antibody specificity in experimental autoimmune myasthenia gravis [9, 12]. Lastly, we are using these mAbs to determine the specificities of anti-AChR autoantibodies in myasthenia gravis patients by studying competitive binding of mAbs and autoantibodies to AChR from human muscle [16]. For all of these purposes, our library of anti-AChR mAbs is valuable only to the extent that we can map the specificities of the mAbs in it.

Previously we have taken several approaches to mapping the specificities of anti-AChR mAbs including competitive binding of mAbs to intact AChR [9, 10], characterizing the complexes of AChR with mAbs by sucrose-gradient centrifugation [14], and testing for binding to ^{125}I-labeled denatured AChR subunits [9, 10] and to proteolytic fragments of subunits [13]. In this paper we test for binding to proteolytic fragments of all four AChR subunits from Torpedo. Carried to its natural limits in the future, this approach should permit us to map the binding sites for many mAbs precisely to the amino acid sequences of the subunits. Much is to be learned even at this stage simply by determining, as we do here, a minimum estimate of the number of antigenic determinants and their distribution over the subunits of the AChR molecule.

MATERIALS AND METHODS

AChR from Torpedo californica electric organ was purified by affinity chromatography on toxin-agarose columns [20]. Subunits were isolated by preparative gel electrophoresis and iodinated using the immunobilized lactoperoxidase method. The specific activities obtained were 1–3 $\times$ 10^{18} cpm mol^{-1}. Ten microliters of each

ammonium sulphate concentrated mAb solution (approximately 200 μg/ml immunoglobulin) was added to an aliquot of an iodinated subunit (90 μl of 10 mM Na phosphate buffer, pH 7.5, containing approx. 3×10^{-12} moles at 4×10^{-8} M of each subunit, total cpm 2×10^6). After overnight incubation, 25 μl of goat antirat antibody coupled to Sepharose (10 mg affinity-purified antibody/ml Sepharose) was added and the mixture shaken at 4°C for 2 hr. The Sepharose-antibody complex was washed with 1 ml of 10 mM Na phosphate, 100 mM NaCl 0.5% Triton X-100, pH 7.5, five times and then twice with 1 ml of water. The bound subunit was solubilized in 2% SDS and the radioactivity determined. The "percent binding" figure is the counts bound by a particular mAb as a percentage of the radioactivity bound by 5 μl of an antisubunit serum (titer $\simeq 5 \times 10^{-6}$ M) minus the background obtained with normal rat serum.

Subunit fragment mapping was performed as follows: Two volumes of ^{125}I-labeled subunit (specific act. 1–3 $\times 10^{18}$ cpm mol^{-1}, 2×10^{-7} M in 10 mM Na phosphate buffer, pH 7.5, containing 0.1% SDS) were mixed with one volume of V8 protease solution (in 125 mM Tris HCl buffer pH 6.8 containing 0.1% SDS) and incubated for 30 min at 23°C. The α subunit was digested with 1 mg/ml protease and the other three subunits with 0.1 mg/ml protease to obtain suitable sized fragments [13]. The reaction was terminated by heating the solution to 100°C for 5 min. The solution was then diluted to 3×10^6 cpm/100 μl (approx. 5×10^{-8} M) in 0.1% SDS, 0.5% Triton X-100. To this (90 μl) was added 10 μl of mAb, 5 μl of antisubunit serum, or 5 μl of normal rat serum. The incubation and addition of second immobilized antibody and washing was as described above. The radioactive fragments dissociated by the SDS treatment were counted and equal cpm were applied to each lane of a 15% polyacrylamide SDS gel which was electrophoresed at 35 mA for 2½ hr at 23°C using two cooling fans. The gels were fixed, dried, and autoradiographed for 2–48 hr at −70°C using Kodak XAR5 film, preflashed to 0.125 absorbance units, and an intensifying screen [21].

RESULTS

mAbs were produced by hybridomas obtained by fusing mouse myeloma cells with spleen cells from rats immunized with either native AChR from Torpedo californica electric organ [9], Electrophorus electricus electric organs [10], bovine muscle, or human muscle [12]. mAbs were also obtained from rats immunized either with a mixture of SDS-denatured subunits of the AChRs from Torpedo [9] or Electrophorus [10] or with isolated individual subunits from Torpedo AChR [9, 12]. In all, 154 cloned antibodies were tested for their ability to bind to individual intact iodinated subunits from Torpedo AChR (Fig. 1). When the immunogen was native AChR from Torpedo, 42% of the mAbs produced reacted detectably with the denatured subunits of Torpedo AChR (5/12). Four mAbs reacted with the α subunit, three with β, none with γ, and one with δ. When the immunogen was a native AChR from a species other than Torpedo (ie, Electrophorus, bovine, or human), only 12% of the mAbs produced reacted with denatured subunits of Torpedo AChR (7/58). When the immunogen was denatured AChR or individual subunits of Torpedo AChR, 80% of the mAbs reacted with denatured subunits of Torpedo AChR (67/84). Generally, those

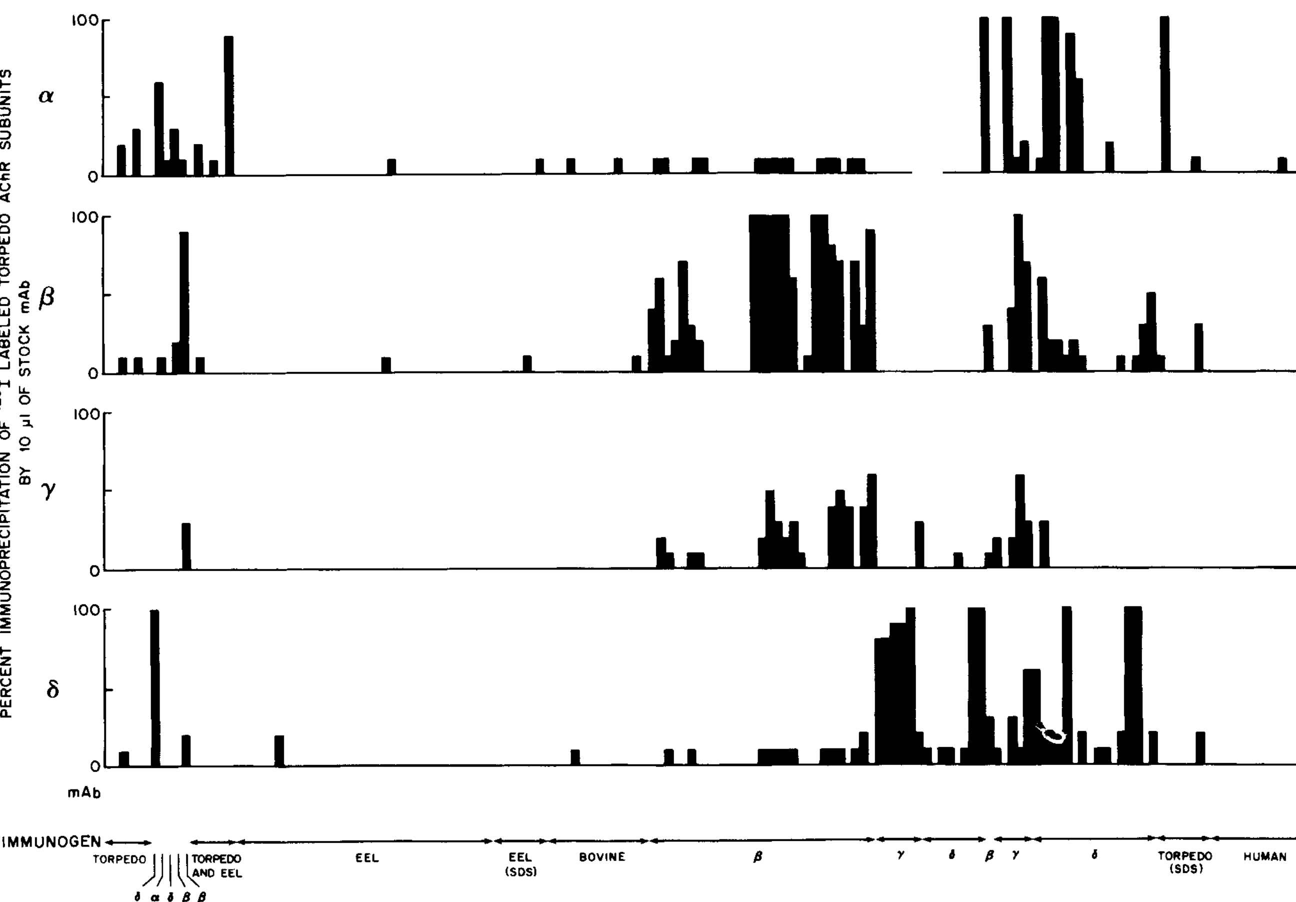

Fig. 1. Immunoprecipitation of individual iodinated subunits of the AChR from Torpedo by mAbs.

mAbs derived from animals immunized with denatured AChR reacted more strongly with denatured material than did those derived from animals injected with native AChR molecules.

In order to distinguish antigenic determinants within subunits, the denatured ^{125}I-labeled subunits were cleaved into relatively large overlapping peptide fragments with V8 protease and the pattern of peptides bound by each mAb was determined (Fig. 2). We had previously applied this method to just the α subunit from Torpedo, and found that mAbs fell into groups according to the pattern of fragments which they bound [13] and that these same groups of mAbs were distinguished by competitive binding to the intact AChR molecule [9] or the types of complexes they form with intact AChR [14]. This suggests that each group of peptide fragments indicates the presence of at least one antigenic determinant. Thirty mAbs to the α subunit were divided into seven groups by their fragment binding patterns (Fig. 3). Two "groups" contained only one mAb that gave unique binding patterns. Forty-six mAbs were mapped against fragments of the β subunit, and these fell into five groups, one of

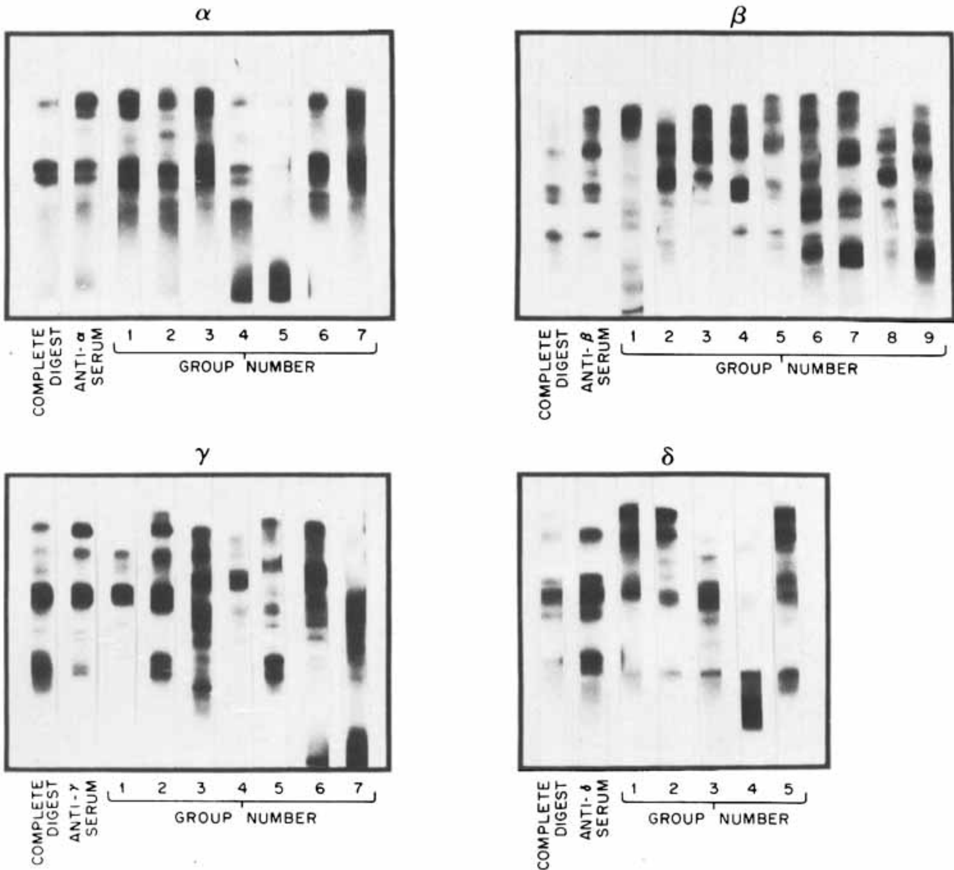

Fig. 2. Immunoprecipitation of V8 protease-generated fragments of iodinated subunits of the AChR from Torpedo by representatives of each mAb group. The fragments were derived as described in Methods, and were separated on 15% polyacrylamide gels containing SDS and visualized by autoradiography.

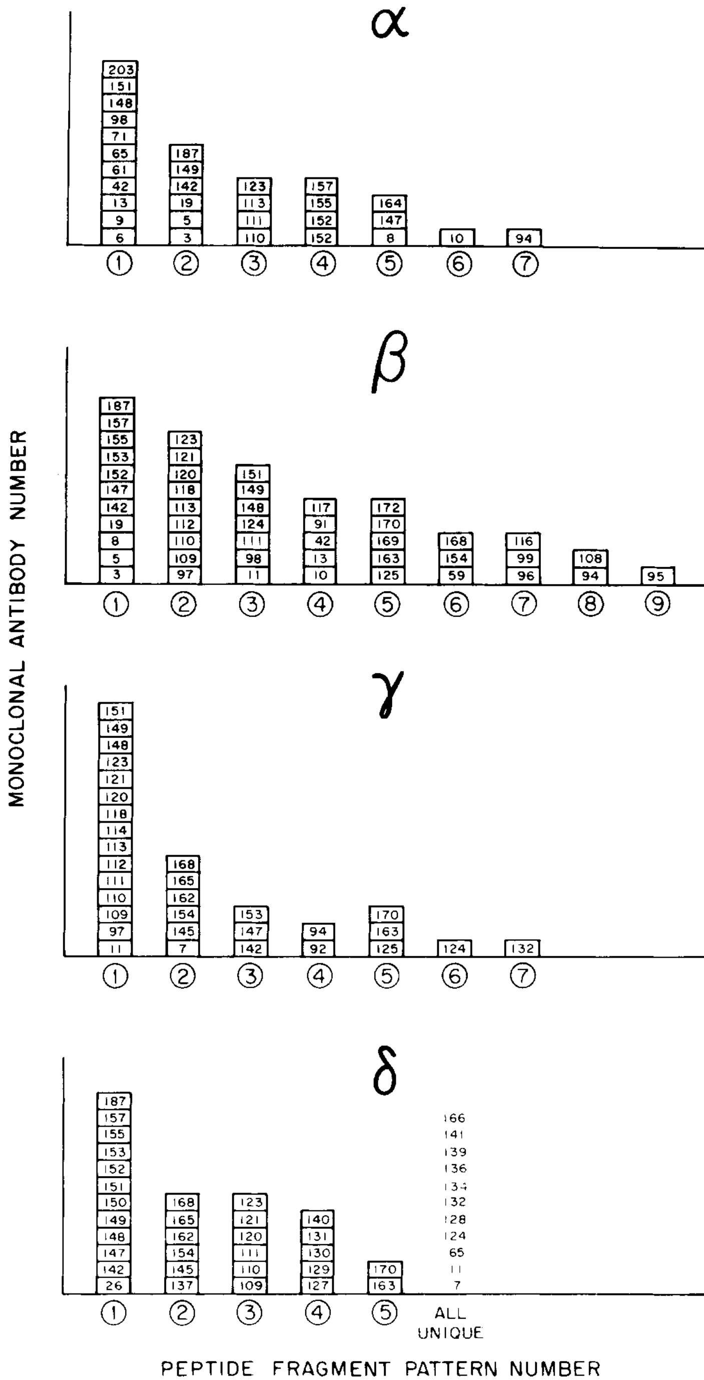

Fig. 3. Grouping of mAbs according to their binding patterns of V8 protease-generated fragments of iodinated subunits of the AChR from Torpedo.

which was represented by a single mAb. Thirty-one mAbs bound to fragments of the γ subunit, forming seven groups, two of which contained one mAb. Finally, 42 mAbs bound to fragments of the δ subunit. In this case 11 mAbs gave unique fragment patterns, but the remaining 31 formed five groups.

Many mAbs crossreact between subunits. This was initially recognized with a few mAbs [9] and suggested structural homologies between AChR subunits that were later confirmed by partial amino acid sequence analysis [5]. Group 3 against α, for instance, contains mAbs that react as group 2 against β, as group 1 against γ, and as group 3 against δ. Alternatively, some mAbs are quite subunit-specific such as group 4 against δ. Of the 74 mAbs tested, 32 reacted detectably with only one subunit, 19 with two subunits, 15 with three subunits, and eight with all four subunits.

DISCUSSION

Previously, we had been able to identify three sites on denatured α subunits at which monoclonal antibodies could bind [13]. Now we can recognize four more sites. In addition, we have recognized at least three other sites on α that are absolutely conformationally dependent so that monoclonal antibodies to them do not react detectably with denatured α [14]. Here we have described at least 21 potential antigenic determinants recognizable on denatured β, γ, and δ subunits. No doubt there are a number of conformationally dependent antigenic determinants on these subunits as well. The demonstration that there are in excess of 30 determinants identifiable on the AChR molecule indicates that immunodissection of its structure should be possible at fairly high resolution. Future studies will determine which of these determinants are involved in AChR function by testing which mAbs can block AChR function. Future studies will also determine the pathological significance of these antibody specificities by testing which antibodies can fix complement, which can cause antigenic modulation of AChR, and which can passively transfer experimental autoimmune myasthenia gravis.

All antigenic determinants are not equivalently immunogenic. We have previously shown that there is a conformationally dependent "main immunogenic region" on the extracellular surface of the α subunits of AChRs from all species examined at which most of the antibodies in the sera of an animal immunized with intact AChR (but not denatured AChR) are directed [9, 10]. Furthermore, we showed that patients with myasthenia gravis and animals immunized with AChR both produce a similar spectrum of antibody specificities [16]. In both cases, although many antibodies are made to the main immunogenic region, the response is clearly polyclonal. Furthermore, the observations that immunization with any of the AChR subunits can induce experimental autoimmune myasthenia gravis [20] and that there is no detectable correlation between the spectrum of anti-AChR specificities produced by myasthenia gravis patients and the severity of their muscular weakness [16] shows that there is no single "myasthenogenic" determinant on the AChR. Nonetheless, some antigenic determinants may be more pathologically significant than others. Defining the determinants as we have done here is clearly the first step toward determining their pathological significance.

Immunization with even a very simple antigenic determinant such as a hapten produces antibodies of several distinguishable idiotypes [22]. The antigenic determi-

nants on a protein are both larger and chemically and conformationally more complex than a hapten, so a much larger family of idiotypes will be produced. This is especially true for a large and antigenically complex protein like the AChR. It is thus unlikely that antiidiotype immunosuppressive therapy will be effective. As an example, Lennon and co-workers [23] have clearly shown that immunization against a mAb probably directed at the main immunogenic region produced antiidiotype antibodies, but did not prevent the induction of experimental autoimmune myasthenia gravis in response to a subsequent injection of AChR. Our demonstration of the complexity of the antigenic structure of the AChR (here) and of the polyclonal nature of the immune response to AChR in myasthenia gravis [16] make it unlikely that even a more complex idiotype immunogen would produce an effective immunosuppressive therapy.

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