Monoclonal Antibodies as Probes of Acetylcholine Receptor Structure. 2. Binding to Native Receptor†

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ABSTRACT: Binding of monoclonal antibodies to *Torpedo californica* acetylcholine receptor monomers solubilized in Triton X-100 was studied by centrifugation on sucrose gradients. Antibodies to α subunits were of two types. One type formed complexes of one antibody and one receptor monomer, independent of antibody/receptor ratio. We conclude that the binding sites for these antibodies are oriented on the two α subunits per monomer in such a way that each could be bound by one of the two binding sites of a single immunoglobulin molecule. Most antibodies were of this type. The other type of monoclonal antibody formed complexes of several sizes, including antibody cross-linked receptors, depending on the ratio of antibody to receptor. We conclude that the binding sites for these antibodies are oriented in such a way that the two α subunits per monomer could not be cross-linked by a single antibody molecule. A monoclonal antibody of this type raised against *Electrophorus electricus* receptor was used to show that this receptor also has two α subunits per monomer. This antibody cross-reacted with receptor from fetal calf muscle and was able to induce antigenic modulation of receptor in muscle cells in culture. This suggests that muscle receptor also has two α subunits and that the antibody can cross-link receptor in the plane of the membrane, as it does in solution, and thereby form complexes which enhance endocytosis and increase the rate of receptor destruction. The rate of antigenic modulation decreases at high antibody/receptor ratios, as expected if un-cross-linked complexes of two antibodies and one receptor were not destroyed at a faster rate. Antibodies which cross-link α subunits within a receptor monomer are frequent but would not be expected to be able to induce antigenic modulation. This provides one mechanism by which antisera of equivalent antireceptor titer might differ in their ability to induce antigenic modulation. An antibody which binds to denatured δ and γ subunits forms complexes of only one antibody and one receptor monomer, independent of antibody ratio, as do antibodies thought to cross-link the two α subunits in a monomer. It apparently cross-links δ and γ subunits within the monomer. Some of the monoclonal antibodies to α subunits can bind simultaneously to receptor, while the binding of others is mutually exclusive.

Monoclonal antibodies (mAbs)[1] can, in principle, be prepared to many parts of the surface of the acetylcholine receptor (AcChR) molecule and thereby provide a large number of specific probes for AcChR structure and function. In preceding papers, we described the preparation of cloned hybridoma cell lines which secrete mAbs to AcChR (Tzartos & Lindstrom, 1980, 1981) and methods for mapping the specificities of these antibodies to individual AcChR subunits (Tzartos & Lindstrom, 1980, 1981) and, more precisely, to peptide fragments from these subunits (Gullick et al., 1981). Most of these mapping techniques used polypeptide chains denatured in $NaDodSO_4$. However, binding of most antibodies to native AcChR depends largely or entirely on the tertiary conformation of the native antigenic determinant (Lindstrom et al., 1978, 1979b; Tzartos & Lindstrom, 1980, 1981). In this paper, we characterize the kinds of complexes formed between several mAbs and AcChR monomers solubilized in Triton X-100 by separating the various sized complexes through centrifugation on sucrose gradients.

Because mAbs are expected to be directed at a single antigenic determinant, they can form characteristic stoichiometric complexes with their antigen. The size of these complexes can provide information about the number and orientation of antigenic determinants within the antigen molecule. Such experiments are difficult or impossible with conventional antisera. For example, antisera to AcChR subunits contain antibodies to many antigenic determinants within each subunit (Gullick et al., 1981), and there are no doubt several antibody species present differing in affinity for each antigenic determinant. Torpedo AcChR monomers consist of two α subunits and one each of β, γ, and δ (Reynolds & Karlin, 1978; Damle & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980). Therefore, as shown in Figure 1A, one would expect that a mAb to an antigenic determinant present only once in the molecule, like a unique determinant of β, γ, or δ, could form complexes only of one antibody and one AcChR monomer in antibody excess or of one antibody and two AcChR monomers in AcChR excess. An antigenic determinant present in two copies, such as one on α subunits, presents a more interesting case. As shown in Figure 1B, if these two determinants were present in a spacing and orientation which permitted both binding sites of an IgG molecule to bind simultaneously within the monomer, only complexes of one antibody and one AcChR monomer could be formed, regardless of the amount of antibody added. Because the diameter of the AcChR molecule (~90 Å; e.g., Zingsheim et al., 1980) is less than the maximum distance which can be bridged by the two binding sites of the IgG molecule (120 Å; e.g., Valentine & Green, 1967), it is likely that the orientation of two antigenic determinants on the surface of the AcChR molecule is more critical than the space between them in determining whether the two antigenic determinants can be bridged by a single IgG molecule. If two identical determinants were oriented on the AcChR monomer so that they could not be bridged by a single anti-

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[1] Abbreviations used: AcChR, acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; eel, *Electrophorus electricus*; [^{125}I]αBGT, ^{125}I-labeled α-bungarotoxin; mAb, monoclonal antibody; MG, myasthenia gravis; $NaDodSO_4$, sodium dodecyl sulfate; torpedo, *Torpedo californica*; IgG, immunoglobulin G; F(ab), monovalent antibody fragment; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium iodide.

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body molecule, Figure 1C shows that several sizes of complex would be formed, depending on the ratio of antibody to AcChR. In AcChR excess, complexes of one antibody and two AcChR molecules would be formed. At equivalence, these complexes and others including precipitates would coexist. In antibody excess, characteristic complexes of two antibodies and one AcChR would be formed.

In this paper, we characterize mAbs to torpedo AcChR α subunits of both types shown in Figure 1B,C. We term these intramolecular or internal cross-linking antibodies when both binding sites of an antibody molecule can bind simultaneously on two determinants within one AcChR monomer and intermolecular cross-linking antibodies when the antibody cannot cross-link the two determinants. Our studies confirm the existence of two α subunits per AcChR monomer in torpedo AcChR and suggest this same stoichiometry in AcChR from eel electric organ and mammalian muscle. We provide data which will help map the orientation of the antigenic determinants of these mAbs in the AcChR monomer. Further, mapping of antibody specificity in this way should permit prediction of the effect of bound antibodies on AcChR metabolism. It is known that antisera to AcChR increase the rate of AcChR degradation in cells (Heinemann et al., 1977; Kao & Drachman, 1977) by cross-linking AcChR (Drachman et al., 1978; Lindstrom & Einarson, 1979) into aggregates (Prives et al., 1979) which presumably promote endocytosis and are known to result in lysozomal destruction of AcChR (Merlie et al., 1979a,b). This is termed antigenic modulation. It would be expected that intramolecular cross-linking antibodies could not cause antigenic modulation whereas intermolecular cross-linking antibodies could. We report that a mAb which can intermolecularly cross-link AcChR is, in fact, capable of inducing antigenic modulation.

Materials and Methods

AcChR was purified by affinity chromatography as previously described (Lindstrom et al., 1978, 1980a). AcChR from *Electrophorus electricus* (eel) was obtained in monomeric form, while primarily dimers were obtained from *Torpedo californica* (torpedo). Torpedo AcChR were converted completely to monomers by reduction (Chang & Bock, 1977) followed by alkylation to prevent reassociation. AcChR dimers were dialyzed against 10 mM sodium phosphate buffer, pH 8.5, containing 0.5% Triton X-100 and 100 mM NaCl. The dialyzed solution was made 1 mM in dithiothreitol. After 1 h at 4 °C, 0.1 volume of 10 mM iodoacetamide was added in 100 mM sodium phosphate buffer, pH 7.5. AcChR was trace labeled with 0.25 mol equiv of ^{125}I-labeled α-bungarotoxin ([^{125}I]αBGT), and monomers were separated from remaining dimers by centrifugation on sucrose gradients. For this purpose, 200 μL of reduced and alkylated AcChR labeled with [^{125}I]αBGT was loaded on 4.9 mL of 5–20% linear sucrose gradients containing 10 mM sodium phosphate buffer, pH 7.5, 0.5% Triton X-100, and 100 mM NaCl. After centrifugation in a Beckman VTi65 rotor at 55 000 rpm for 75 min at 5 °C, fractions of 135 μL were collected from the bottom of the tube, and ^{125}I was measured in a γ counter.

Preparation and some of the properties of most of the mAbs used have been previously described (Tzartos & Lindstrom, 1980, 1981; Lindstrom et al., 1980b; Gullick et al., 1981). Size of the mAbs [7 S (e.g., IgG) or 19 S (IgM)] was determined by centrifugation on sucrose gradients of 5–20% sucrose as above in a Beckman SW50.1 rotor for 5 h at 50 000 rpm at 5 °C. Fractions were assayed for antibody activity.

Antibody concentration was measured as previously described by using antigen in excess at a concentration of 1×10^{-9} M (Lindstrom et al., 1979a). AcChR concentration was similarly determined by immune precipitation (Lindstrom et al., 1978). The ratio of mAb molecules per AcChR monomer was calculated from the mAb and AcChR titers in moles of [^{125}I]αBGT binding sites bound per liter. It is assumed that each AcChR monomer binds two [^{125}I]αBGT molecules, that 75% of the AcChR in the mAb assay are dimers, and that in the AcChR excess conditions of the mAb assay both binding sites of the antibody are occupied. Thus, molar concentration of mAb molecules is approximately the titer divided by six in the case of mAb molecules which can intermolecularly cross-link AcChR and approximately the titer divided by three in the case of mAbs which cross-link subunits within an AcChR monomer.

Complexes between mAbs (at various concentrations) and [^{125}I]αBGT-labeled AcChR monomers (typically 5×10^{-9} M) were made by overnight incubation at 5 °C in mAb/AcChR ratios from 1:4 to 100:1. Aliquots of 100 μL were layered on the sucrose gradients described above. The gradients were spun in a Beckman SW50.1 rotor at 50 000 rpm for 5–9.5 h, depending on the resolution desired. [^{125}I]αBGT was measured in 135-μL (14-drop) fractions. Presence of bound mAb in the peaks was demonstrated by adding normal rat serum (5 μL) as carrier and precipitating with goat antirat Ig (cf. Lindstrom et al., 1976). [^{125}I]αBGT in the immune precipitate and supernatant was determined.

For determination of whether different mAbs could bind simultaneously to AcChR or compete for the same or nearby binding sites, aliquots of 1×10^{-8} M AcChR labeled with [^{125}I]αBGT were incubated overnight at 5 °C with a 100-fold excess of mAb. Then a 100-fold excess of the second mAb was added for a further 4–12 h. Finally, samples were centrifuged on sucrose gradients as described above.

AcChR subunits were purified and iodinated as previously described (Lindstrom et al., 1979a). After incubation at 10^{-8} M with mAb, 100-μL aliquots were centrifuged on gradients in the SW50.1 rotor for 17 h at 50 000 rpm. In some experiments, both samples and the gradient solutions contained 0.1% $NaDodSO_4$ and 1 mM dithiothreitol.

Antigenic modulation was measured essentially as previously described (Heinemann et al., 1977; Lindstrom & Einarson, 1979). Primary cultures of fetal bovine muscle cells were plated for 11 days prior to the experiment. AcChR on the cells was labeled by 30-min incubation with 10^{-8} M [^{125}I]αBGT, and then unbound [^{125}I]αBGT was removed by changing the medium several times. Medium or medium supplemented with mAb was then added. At intervals, the medium was replaced by medium with or without mAb, and ^{125}I released into the medium was determined. At the end of the experiment, ^{125}I still associated with the cells was determined to give a 100% value.

Results

Torpedo AcChR normally exists as dimers linked by a disulfide bond between δ subunits on each monomer (e.g., Chang & Bock, 1977). For simplification of the analysis, dimers were cleaved by reduction and the monomers purified by sucrose gradient centrifugation. Each monomer was expected to consist of two α subunits and one each of β, γ, and δ (Reynolds & Karlin, 1978; Lindstrom et al., 1979b; Raftery et al., 1980). Because it was known that [^{125}I]αBGT did not compete for the binding sites of the mAbs used (Tzartos & Lindstrom, 1980), [^{125}I]αBGT-labeled AcChR monomers were used as antigen.

Polyclonal antisera to native AcChR and its denatured subunits (Lindstrom et al., 1978, 1979a) formed large

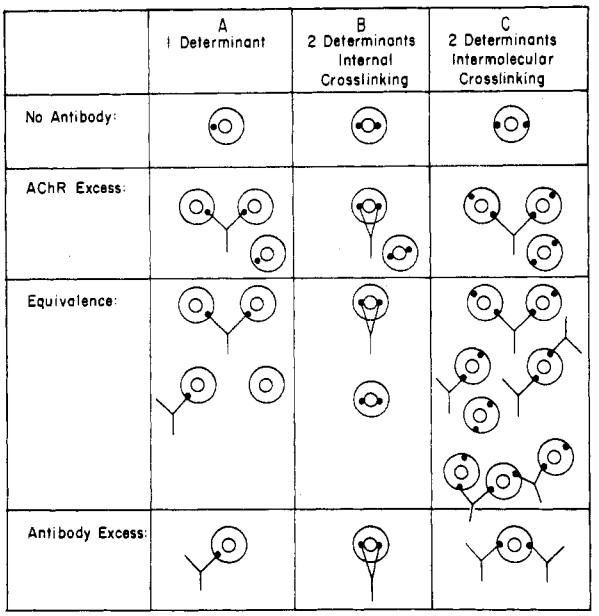

FIGURE 1: Kinds of complexes which can be formed between monoclonal antibodies and AcChR monomers.

amorphous aggregates with labeled AcChR monomers that pelleted on sucrose gradients (data not shown). Even in 100-fold excess, antisera to α, β, γ, or δ subunits did not form small characteristic antibody–AcChR complexes of the types described in Figure 1. This was anticipated from the observation that antisera to any of the subunits could bind all the proteolytic fragments of the subunit (Gullick et al., 1981), showing that there are many antigenic determinants recognized by these sera on each denatured subunit polypeptide chain.

mAbs to native AcChR and its denatured subunits form characteristic stoichiometric complexes with AcChR monomers of the types shown in Figure 1. Some properties of the mAbs used in these studies are summarized in Table I. mAbs of 7S size were selected for further study. Antibodies of 7 S were used because they would be expected to be bivalent IgG and much simpler to characterize than decavalent 19S IgM. mAbs to α were used for most studies. We used 15 mAbs in the experiments reported here of 70 mAb which we have prepared to date. Preparation of mAbs 1–19 was described by Tzartos & Lindstrom (1980), and the others will be described elsewhere. Tzartos & Lindstrom (1980) determined the subunit binding specificity by mAb binding to purified denatured subunits and by competitive mAb binding to native AcChR conjugated to agarose. The binding sites of some of these mAbs which bound detectably to denatured subunits were more precisely mapped by using peptide fragments of the subunits by Gullick et al. (1981). Here, we investigated the binding of mAb to Triton X-100 solubilized AcChR monomers by using sucrose gradient centrifugation. Because the molecular weight of IgG (150000) is substantial with respect to AcChR monomers (250000) (Reynolds & Karlin, 1978), complexes of one or two mAbs and one or two AcChR should be well resolved.

mAb 6 is a high-affinity antibody which cross-reacts with AcChR from many species (Tartos & Lindstrom, 1980). It binds to a conformationally dependent antigenic determinant but still reacts slightly with denatured α and the MBTA-labeled peptide fragment of the α subunit (Gullick et al., 1981). Its binding site is sufficiently far from the MBTA binding site that its binding is not inhibited by α-bungarotoxin. The area around its binding site is highly immunogenic and accounts for about 50% of the antibodies in an antiserum to native

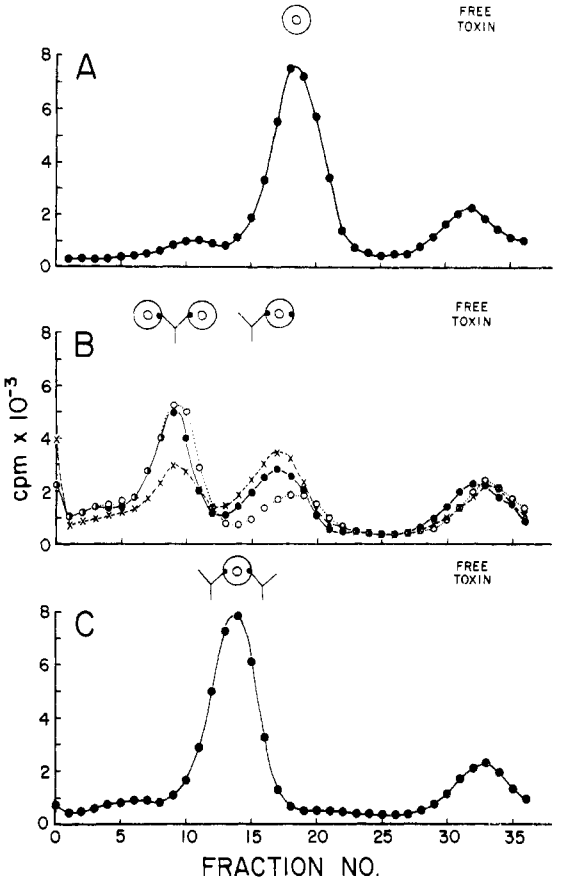

FIGURE 2: Binding of mAb 6 to torpedo AcChR monomers at increasing antibody/AcChR ratios. Sucrose gradients using [^{125}I]-αBGT-labeled AcChR monomers were centrifuged simultaneously after overnight incubation of mAb and AcChR as described under Materials and Methods. The symbols over the peaks indicate the type of mAb–AcChR complexes thought to account for the observed peaks. (A) Purified monomers. Traces of dimer peak in fraction 11. (B) Monomers plus mAb 6. Antibody/AcChR = 1:2 (○), antibody/AcChR = 1:1 (●), antibody/AcChR = 2:1 (×). (C) Antibody in 100-fold excess over AcChR.

AcChR (Tzartos & Lindstrom, 1980, 1981). This antibody forms complexes of the type described in Figure 1C, suggesting that there are two antigenic determinants per AcChR monomer located in an orientation which prevents internal crosslinking. Figure 2A shows AcChR monomers. Figure 2B shows that addition of increasing amounts of mAb 6 in AcChR excess causes the disappearance of monomers and the gradual appearance of complexes of one mAb and one AcChR, one mAb and two AcChR, and large aggregates and precipitate. The amount of AcChR precipitated by mAb 6 (calculated as the amount in the gradient pellet plus the first four fractions) ranged from 7% to 20% when antibody and AcChR were near equivalence and decreased to 2–3% when antibody was in 100-fold excess. The amount of AcChR precipitated by mAb 6 is probably limited because only linear rather than branched polymers can form between a bivalent antigen and a bivalent antibody.

Figure 2C shows that in antibody excess only a single complex of the size expected of two mAbs and one AcChR is observed. By immune precipitation it was demonstrated that AcChR in the peaks thought to contain antibody–AchR complexes could, in fact, be precipitated by goat antirat Ig. The results shown in Figure 2 are typical of many similar experiments with mAb 6. When the positions of AcChR monomers and dimers are used as standards, the size of complexes of mAb and AcChR follow the order expected in Figure 1C. Identification of the mAb–AcChR complexes formed is

Table I: Properties of Monoclonal Antibodies Used

mAb[a]	immunogen	Ig class[b]	subunit specificity[c]	titer against native AcChR (apparent mol of mAb/L)[c,d]	titer against denatured subunit (apparent mol of mAb/L)[c,d]	conformation dependence[e]	competition with other antibodies[c]	V8 peptide fragments[f] of α bound	cross-linking
1	torpedo AcChR	7 S	α	12.3×10^{-6} M	0	100%	2, 4, 6, 12, 16	none	internal
2	torpedo AcChR	7 S	α	10.4×10^{-6} M	0	100%	1, 4, 6, 12, 16	none	internal
3	torpedo AcChR	7 S	α	4.3×10^{-6} M	1.1×10^{-7} M	97%	5, anti-α sera	VD, VG, VH	internal
4	torpedo AcChR	7 S	α	4.4×10^{-6} M	0	100%	1, 2, 4, 16	none	
5	torpedo AcChR	7 S	α	13.9×10^{-6} M	1.6×10^{-6} M	89%	3, anti-α sera	VD, VG, VH	internal
6	torpedo AcChR	7 S	α	8.0×10^{-6} M	1×10^{-10} M	>99%	1, 2, 4, 14, 16	VA, VB, VC, VD, VE (MBTA labeled) VF (contains carbohydrate)	intermolecular
7	torpedo δ	7 S	δ (also γ)	1.8×10^{-6} M	4.5×10^{-7} M (δ) 2.3×10^{-7} M (γ)	75%	anti-δ sera		internal
8	torpedo α	7 S	α	3.3×10^{-6} M	8.3×10^{-8} M	98%	anti-α sera	VD, VH, VI	internal
10	torpedo β	7 S	β (also α)	$<7.4 \times 10^{-8}$ M	2.2×10^{-9} M (α) 4.8×10^{-9} M (β)	~97%			
11	torpedo β	19 S	β	$<2 \times 10^{-6}$ M	$<2.6 \times 10^{-6}$ M	~0	anti-β sera		
12	torpedo AcChR	7 S	α	6.1×10^{-6} M	0	100%	1, 2, 16	none	internal
13	torpedo AcChR	19 S	α	$<7 \times 10^{-8}$ M	1.1×10^{-9} M	~98%			
14	torpedo AcChR	7 S	α	13.9×10^{-6} M	0	100%	1, 4, 16	none	internal
35	eel AcChR	7 S	α of eel	3.7×10^{-6} M (eel)	n.t.	nt	6[d]	none	intermolecular
				1.2×10^{-6} M (torpedo)	0	100%			
37	eel AcChR	7 S	α of eel	1.5×10^{-6} M (eel)	n.t.	nt	6[d]	none	intermolecular
				8.3×10^{-7} M (torpedo)	0	100%			

[a] mAb numbers are those assigned in Tzartos & Lindstrom (1980) and used in Gullick et al. (1981). [b] 7S antibodies are presumed to be bivalent IgG whereas 19S antibodies are presumed to be decavalent IgM. [c] Data from Tzartos & Lindstrom (1980). Antibody titers against native torpedo AcChR were divided by 6 to give apparent antibody concentrations of intermolecular cross-linking antibodies and by 3 for intramolecular cross-linking antibodies. Titers against denatured subunits were divided by 2 to give apparent antibody concentration. Titer against native eel AcChR (which exists only as monomers) was divided by 4 to give apparent antibody concentration of intermolecular cross-linking antibodies to eel AcChR. [d] Data from Tzartos et al. (1980). [e] The extent of conformation dependence was calculated as the [[(apparent concentration of mAb to native AcChR) − (apparent concentration of mAb to subunit)]/(apparent concentration of mAb to native AcChR)] × 100%. [f] Data from Gullick et al. (1981).

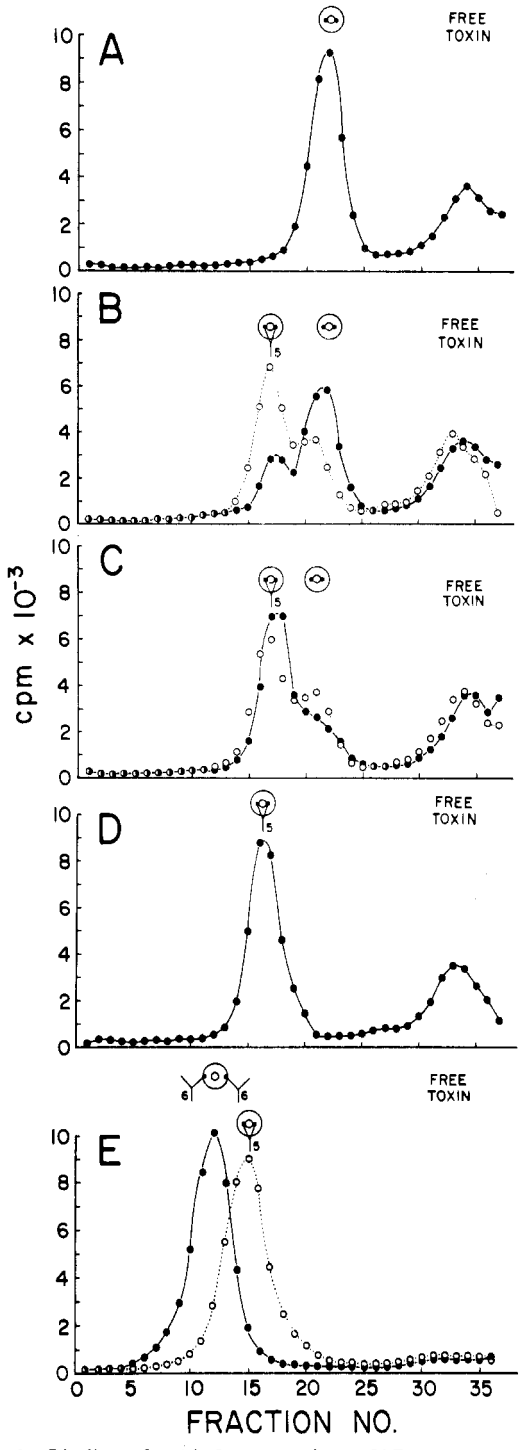

FIGURE 3: Binding of mAb 5 to torpedo AcChR monomers at increasing antibody/AcChR ratios. Sucrose gradients of mAb–AcChR mixtures were centrifuged as in Figure 2. (A–D) were centrifuged in one run, while (E) was in another. The symbol over the peaks indicates the type of complexes thought to account for a 7 at of this size. (A) Purified monomers. (B) Monomers plus mAb 5. Antibody/AcChR = 1:4 (●), antibody/AcChR = 1:2 (○). (C) Monomers plus higher amounts of antibody. Antibody/AcChR = 1:2 (○), antibody/AcChR = 1:1 (●). (D) mAb 5 in 100-fold excess over AcChR. (E) Comparison of complexes formed by mAbs 5 and 6 in 100-fold excess over AcChR. mAb 5 (○), mAb 6 (●).

based on the internal consistency of their relative size at various mAb/AcChR ratios rather than on measure of the absolute size of the complexes.

mAbs 3 and 5, in contrast with mAb 6, produced complexes with AcChR of only a single size, independent of mAb/AcChR ratio. This would be expected of an internal cross-linking antibody of the type depicted in Figure 1B. mAb 5

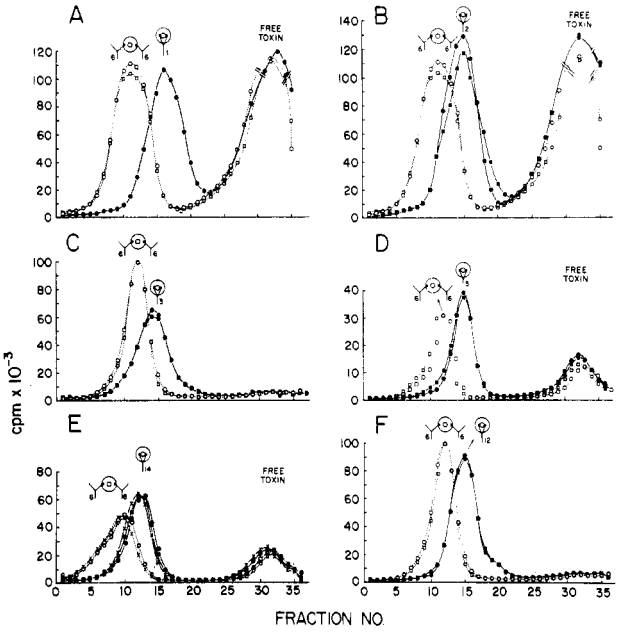

FIGURE 4: Size of complexes formed in antibody excess by several mAbs. In each panel the complexes formed by mAb 6 (○, □, ×) are compared with those formed by the test mAb (●, ■) in the same centrifuge run. Duplicate gradients in many cases show the reproducibility of the method. Symbols over the peaks show the types of complexes thought to compose them. (A) mAb 1 in 100-fold excess. (B) mAb 2 in 100-fold excess. (C) mAb 3 in 100-fold excess. (D) mAb 5 in 100-fold excess. (e) mAb 14 in 1000-fold excess. (F) mAb 12 in 1000-fold excess.

is a high-affinity antibody to α which is very species specific for torpedo (Tzartos & Lindstrom, 1980). It is directed at an antigenic determinant on a peptide distinct from that recognized by mAb 6 (Gullick et al., 1981). mAb 3 has a similar specificity and forms a similar unique complex with AcChR. The concentration dependence of their formation is the same for mAbs 1, 2, 3, 5, and 8 and will be illustrated here only with mAb 5. As increasing amounts of mAb 5 are added to AcChR monomers, only a single species of complex is formed (Figure 3B–D). It is of the same size as the complex attributed to one mAb and one AcChR in the case of mAb 6. This complex is smaller than the complex formed by mAb 6 in antibody excess (Figure 3E).

Figure 4 shows that mAbs 1, 2, 12, and 14 in antibody excess produce a single size of complex with AcChR of the same size as that obtained with mAbs 3 and 5, indicating that they too are internally cross-linking antibodies. This figure illustrates the excellent reproducibility of this centrifugal method when samples run in the same rotor are compared. mAbs 1 and 2 are high-affinity antibodies species specific for torpedo (Tzartos & Lindstrom, 1980). Their binding site is highly conformationally dependent since no binding to denatured AcChR subunits is detectable. Binding of mAbs 1, 2 and 6 is mutually exclusive, so the binding site for mAbs 1 and 2 is probably near that of mAb 6 on α (Tzartos & Lindstrom, 1980). However, the binding sites for mAbs 1 and 2 and for mAb 6 are clearly distinct, because the site for mAb 6 is oriented so that a single antibody cannot cross-link the two sites in a monomer whereas the adjacent binding site for mAbs 1 and 2 is oriented so that a single antibody can cross-link the two sites in a monomer. mAb 12 cross-reacts with AcChR from several species and is a conformationally dependent antibody which does not compete with mAb 6 but does compete with mAbs 1 and 2. Thus, mAb 12 probably binds to α at a site near that for mAbs 1 and 2 and close to the site for mAb 6. mAb 14 is a high-affinity antibody which

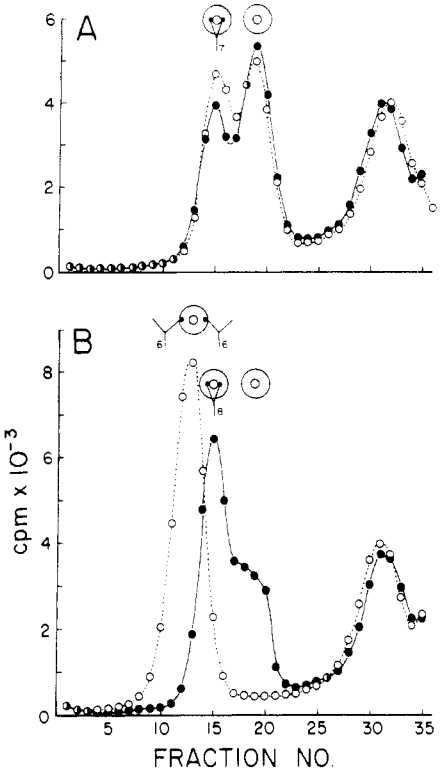

FIGURE 5: Binding of mAbs 7 and 8 to AcChR. Sucrose gradient sedimentation of antibody–AcChR complexes was conducted as in Figures 2–4. In this experiment, 4.8 mL of gradients was centrifuged 8.5 h at 50 000 rpm. (A) Binding of mAb 7 at 1 mAb/AcChR (●) and 100 mAb/AcChR (○). (B) Standards run simultaneously. mAb 6 at 100:1 (○) and mAb 8 at 100:1 (●).

cross-reacts with AcChR from several species (Tzartos & Lindstrom, 1980). It is conformationally dependent. Its binding is not inhibited by mAb 1, 2, 3, 5, 8, or 12 but is inhibited by mAb 16. Because mAb 16 binds to the same peptide fragments of α recognized by mAb 6 (Gullick et al., 1981), it seems that mAb 14 binds to α at a site near mAb 6 but distinct from the sites recognized by mAbs 3 and 5, 1 and 2, 12, or 8. mAb 8 is an internally cross-linking antibody whose binding will be described later. mAb 8 cross-reacts with AcChR from several species (Tzartos & Lindstrom, 1980). It binds to small overlapping peptides from α, some of which include the binding site for mAbs 3 and 5, but the smallest of which is unique to mAb 8 (Gullick et al., 1981). It does not bind to AcChR in membranes, suggesting that its antigenic determinant may normally be partially obscured by membrane lipids (Gullick et al., 1981).

Unlike the mAbs described above, which are directed at α subunits, mAb 7 was prepared against denatured δ subunits. It cross-reacts with γ subunits (Tzartos & Lindstrom, 1980). Raftery et al. (1980) have demonstrated sequence homology between all subunits, but especially between γ and δ. Figure 5 shows that it forms complexes of only one mAb and one AcChR monomer, independent of antibody/AcChR ratio. Thus it behaves like an internally cross-linking antibody. Evidently, it cross-links the single δ subunit in the monomer to the single γ subunit. It has the further interesting property that even at very high antibody/AcChR ratios some of the AcChR monomers do not bind antibody. This may be due to proteolysis during storage. Unpublished observations indicate that proteolysis inhibits the binding of some mAbs but not others.

We investigated the binding of mAbs to denatured ^{125}I-labeled torpedo AcChR subunits by using centrifugation on

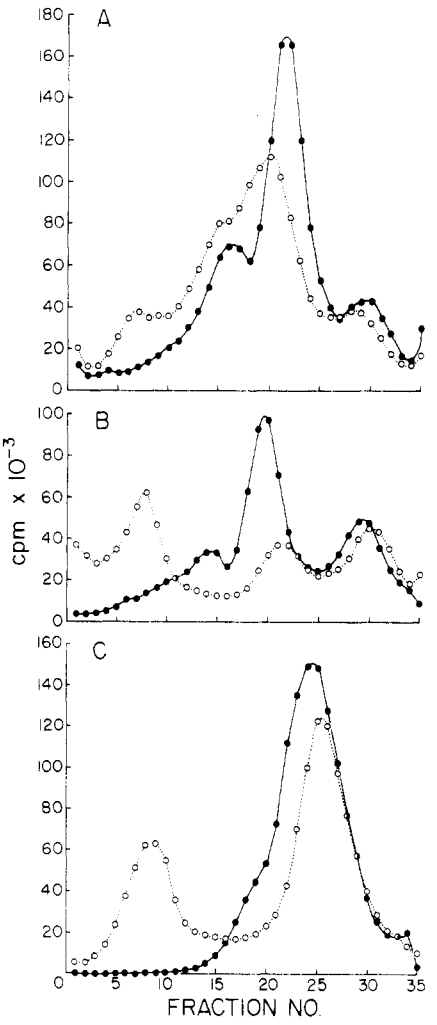

FIGURE 6: Binding of mAbs to ^{125}I-labeled denatured AcChR subunits. Subunits were at 1×10^{-8} M. Gradients were 4.8 mL in 0.5% Triton X-100 as in previous figures, but 0.1% sodium dodecyl sulfate was also added, and gradients were centrifuged for 17 h to resolve the smaller complexes of antibody and denatured subunit. (A) ^{125}Iα (●), ^{125}Iα plus mAb 6 at 300:1 (○), (B) ^{125}Iγ (●), ^{125}Iγ plus mAb 7 at 60:1 (○), (C) ^{125}Iδ (●), ^{125}Iδ plus mAb 7 at 60:1 (○).

sucrose gradients. On electrophoresis in $NaDodSO_4$ on acrylamide gels, purified ^{125}I-labeled subunits migrate as single bands of appropriate apparent molecular weights, and some of the ^{125}I moves with the tracking dye (Lindstrom et al., 1979a, 1980a). On sucrose gradients, it appeared that some or all of the subunits were present as aggregates (Figure 6). This was most pronounced with α and least with δ. The patterns of sedimentation of the individual subunits and the subunits plus mAbs were the same if the gradients contained 0.5% Triton X-100 or if they were supplemented with 0.1% $NaDodSO_4$ or 0.1% $NaDodSO_4$ and 1 mM dithiothreitol. It is possible that the high apparent molecular weights of the subunits might result from some variable degree of association with detergent micelles, but it seems likely that more severe denaturing conditions, such as those during electrophoresis in $NaDodSO_4$, are necessary to prevent some degree of self-aggregation of the subunits. In 0.5% Triton X-100 plus 0.1% $NaDodSO_4$, mAbs can bind to ^{125}I-labeled subunits. Previously, we investigated this by immune precipitation followed by electrophoresis to identify the bound subunit (Tzartos & Lindstrom, 1980, 1981; Lindstrom et al., 1980b; Gullick et al., 1981). Binding of mAb can also be detected by using sucrose gradients (Figure 6). Large excesses of mAbs were used in order to compensate for the low binding affinity for

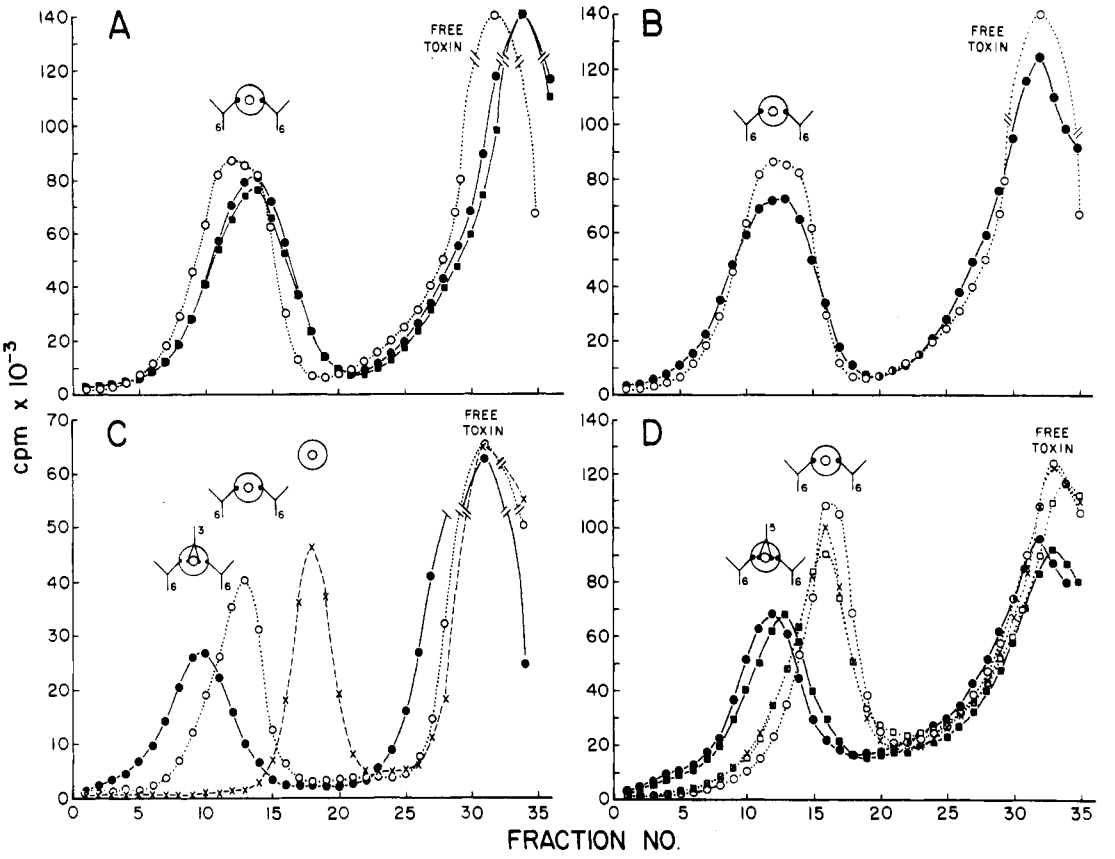

FIGURE 7: Binding of other mAbs to complexes initially formed with AcChR in the presence of excess mAb 6. Aliquots of AcChR were initially incubated overnight with 100-fold excess of mAb 6, and then a 100-fold excess of the test mAb was added for 4–12 h before the complexes were centrifuged on sucrose gradients. In each case, AcChR incubated with mAb 6 alone was run in accompanying gradients as a standard (O, □, ×). In some cases, duplicate gradients with mAb 6–AcChR complexes plus test antibody show the reproducibility of the method (●, ■). (A) plus mAb 1, (B) plus mAb 2, (C) plus mAb 6 alone (O), plus mAb 3 (●), free monomers (×), and (D) plus mAb 5.

denatured subunits and for the long duration of centrifugation. The expected low binding affinity of mAb 6 is especially evident (Figure 6A). Even in 600-fold excess, only part of α is bound, and much of that dissociates during the course of centrifugation. mAb 7 has much higher affinity for denatured subunits, and binding is readily seen (Figure 6B,C). As previously observed (Tzartos & Lindstrom, 1980), we found that mAb 7 cross-reacted with both γ and δ. Note that α and γ, which appear to exist in these solutions mostly as dimers or larger aggregates, form complexes with mAb which trail somewhat toward larger apparent sizes than does δ, which appears to exist in these solutions primarily as dispersed polypeptide chains.

The ability of mAbs of more than one specificity to bind to an AcChR simultaneously was investigated. Not surprisingly, mAb 7, which binds to δ and γ subunits, and mAb 8, which binds to α subunits, could bind simultaneously to AcChR monomers (data not shown). It was more interesting to investigate the binding of mAbs to the several different antigenic determinants on α. The results obtained agreed with the previous experiments (Tzartos & Lindstrom, 1980) in which competitive binding of mAbs was investigated by using AcChR–agarose saturated with one mAb to test whether a second mAb could bind and are consistent with the different binding sites for various mAbs demonstrated by peptide mapping (Gullick et al., 1981). After preincubation with excess mAb 6 followed by incubation with excess mAb 1 or 2, the size of the complex was that characteristic of excess mAb 6 and not that of excess mAb 1 or 2 (Figure 7A,B). Thus, mAb 6 inhibited the binding of mAbs 1 and 2. After preincubation with excess mAb 6 followed by incubation with excess mAb 3 or 5, complexes significantly larger than those characteristic of mAb 6 alone were formed (Figure 7C,D). Thus, mAbs 3 and 5 can form ternary complexes with mAb 6 labeled AcChR. Also, mAb 5 can form ternary complexes with mAb 8 labeled AcChR (Figure 8). Complexes with mAb 3 are not increased in size by addition of mAb 5 (data not shown). Thus, mAbs 3 and 5 are competitive. The binding of mAb 6 and 8 is not competitive (Figure 9). mAb 8 is an internally cross-linking antibody. After binding of mAb 8, the addition of various concentrations of the intermolecular cross-linking mAb 6 (Figure 9C) produces the same pattern obtained when the same ratios of mAb 6 alone are mixed with AcChR monomer (Figure 2), except that all of the complexes are larger by the addition of one mAb 8 per AcChR monomer.

Antigenic modulation is the increase in rate of AcChR destruction in muscle cells triggered by antibody cross-linking of AcChR (Heinemann et al., 1977; Drachman et al., 1978; Lindstrom & Einarson, 1979). Monovalent antibody fragments cannot cross-link AcChR and cannot cause antigenic modulation (Drachman et al., 1978; Lindstrom & Einarson, 1979). It would be predicted that an intermolecular cross-linking mAb like mAb 6 could induce antigenic modulation, while an internally cross-linking mAb like 5 could not. Unfortunately, both of these mAbs are so species specific that their cross-reaction with AcChR in fetal calf muscle cells is too low to be usable. However, mAb 35 was prepared against eel AcChR and cross-reacts well with fetal calf muscle AcChR. It is a 7S antibody whose titer (in moles of [^{125}I]αBGT binding sites bound per liter of serum) against fetal calf muscle AcChR is 3×10^{-6} M. It reacts specifically with denatured eel α subunits (Tzartos & Lindstrom, 1981). mAb 37 is an antibody to eel AcChR which reacts detectably with eel α. Its titer against fetal calf muscle AcChR is 3.8×10^{-7} M. It forms

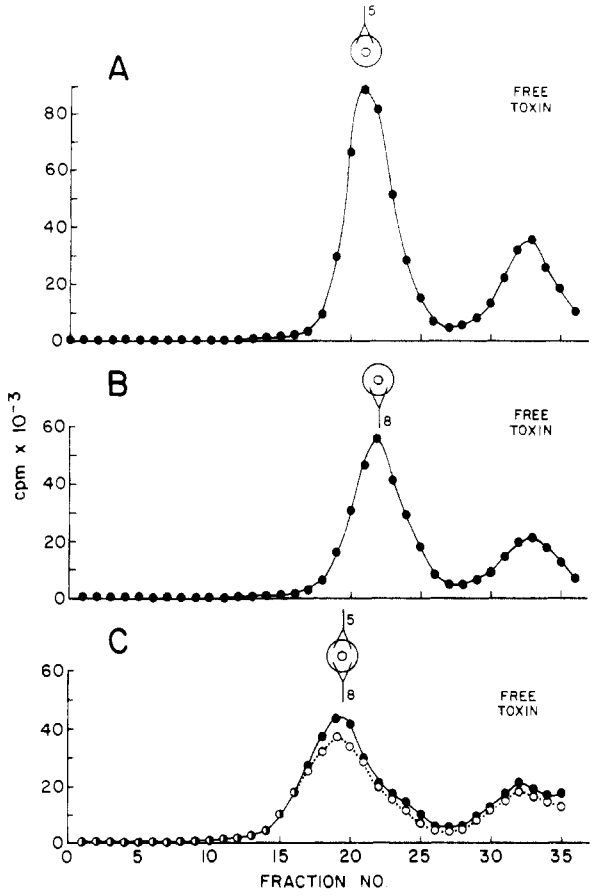

FIGURE 8: Simultaneous binding of mAbs 5 and 8 to AcChR. Sucrose gradients were run as in previous figures. (A) mAb 5 in 100-fold excess. (B) mAb 8 in 100-fold excess. (C) mAb 8 added in 100-fold excess first and then mAb 5 (duplicate gradients).

complexes with eel AcChR identical with those formed by mAb 35. Here, only the complexes formed by mAb 35 will be described in detail. Figure 10 shows that mAb 35 forms complexes with [^{125}I]αBGT-labeled eel AcChR monomers of the same types characteristic of mAb 6 with torpedo AcChR monomers. This result indicates that eel AcChR, like torpedo AcChR, has two α subunits per monomer. It further shows that the antigenic determinant recognized by mAb 35 is oriented like that recognized by mAb 6, so that mAb 35 can form large antibody/AcChR aggregates at appropriate antibody/AcChR ratios. In fact, mAbs 35 and 37 appear to be directed at the region on eel AcChR which corresponds to the region on torpedo AcChR recognized by mAb 6, because mAb 6 competitively inhibits the binding of mAb 35 or 37 to eel AcChR (Tzartos & Lindstrom, 1981, and unpublished results).

Figure 11 shows that mAb 35 can cause antigenic modulation of AcChR in fetal calf muscle, causing an increase in the rate of AcChR turnover from a control $t_{1/2}$ = 21.5 h to a maximum of $t_{1/2}$ = 9.1 h. This suggests that muscle AcChR have two similarly oriented α-subunit determinants through which they can be aggregated in the plane of the membrane by mAb 35. Further support for this idea is the observation that both mAbs 6 and 35 bind to purified Triton X-100 solubilized fetal calf muscle AcChR in the same pattern shown in Figure 2 with torpedo AcChR monomers (Lindstrom et al., unpublished results). mAb 35 also binds to human AcChR in the same pattern observed in Figure 2 (Lindstrom et al., 1981). mAb 1, which is species specific for torpedo AcChR, caused little or no modulation ($t_{1/2}$ = 18.9 h) when equimolar or in 1000-fold excess. Figure 12 shows that the rate of antigenic modulation caused by mAb 35 increased to a plateau

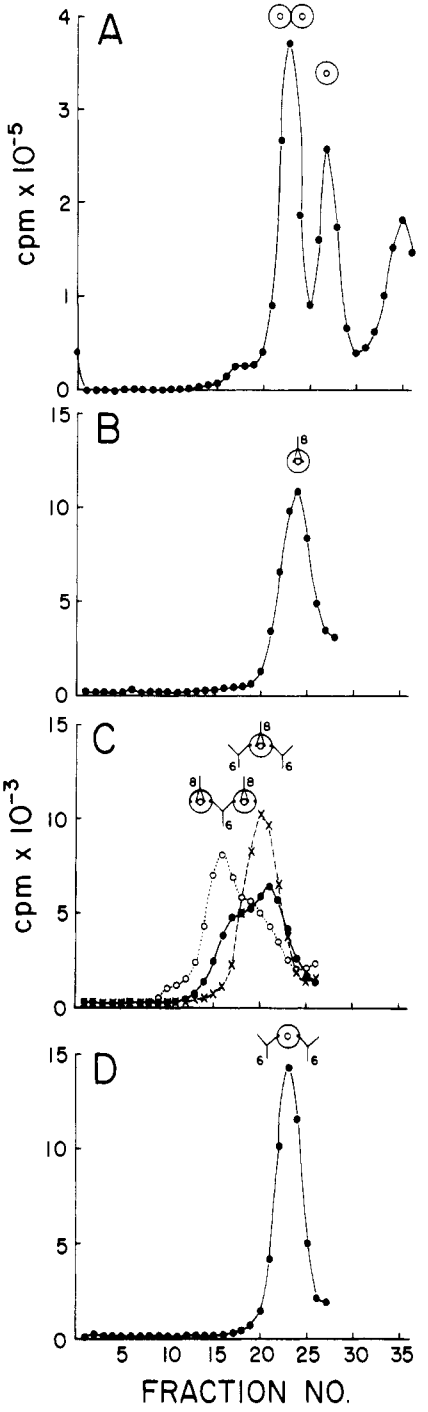

FIGURE 9: Binding of mAb 6 to AcChR monomers with mAb 8 bound. Gradients were run as in previous figures. In this particular experiment, 4.9-mL gradients were run for 5 h at 50000 rpm. (A) AcChR monomers and dimers, for reference. (B) mAb 8 in 100-fold excess. (C) After preincubation with excess mAb 8, mAb 6 was added in calculated ratios of 1:4 (○), 1:2 (●), and 100:1 (×). (D) mAb 6 alone in 100-fold excess.

and then decreased at great antibody excess. These are precisely the results which would be expected if AcChR and antibody in the membrane formed complexes resembling those characterized by ultracentrifugation of soluble antibody-bound AcChR. At lower antibody/AcChR ratios, antibodies apparently cross-linked AcChR into aggregates which facilitated endocytosis (hence, modulation). At high antibody/AcChR ratios, many complexes of the two antibodies per AcChR monomer type were probably formed which did not cross-link AcChR and therefore did not enhance endocytosis and modulation.

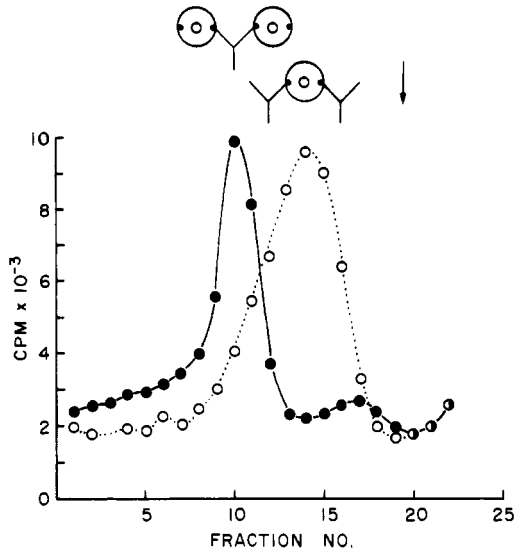

FIGURE 10: Binding of mAb 35 to eel AcChR at increasing antibody/AcChR ratios. As in previous figures, antibody and AcChR were incubated overnight, and then complexes were resolved by centrifugation on sucrose gradients. The position of unbound eel AcChR monomers is indicated by the arrow. Unbound toxin at the top of the gradient is not shown. Antibody/AcChR 1:2 (●). Antibody/AcChR 100:1 (○).

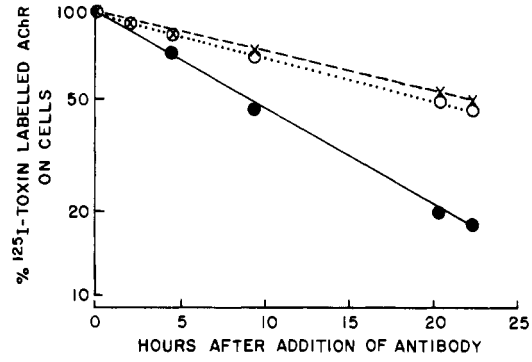

FIGURE 11: Effect of mAbs on the rate of AcChR destruction in muscle cells in tissue culture. AcChR in fetal calf muscle cells were labeled with [^{125}I]αBGT, and then the rate of AcChR destruction was estimated by measuring ^{125}I released into the medium. In the absence of any antibody (×), the half-time of AcChR destruction was 21.5 h. In the continual presence of a 1000-fold excess of mAb 1 (○), which is highly species specific for torpedo α, the rate of destruction was only slightly greater ($t_{1/2}$ = 18.9 h). In the presence of a 10-fold excess of mAb 35 (●), which was prepared against eel AcChR, but cross-reacts with fetal calf AcChR, the rate of destruction greatly increased ($t_{1/2}$ = 9.1 h).

Discussion

In order to understand the interaction of mAbs with AcChR, it is valuable to consider some of the observations from the classic studies of Atassi (1975, 1978), who worked out the complete antigenic structure of the two small soluble proteins myoglobin and lysozyme. Our interest is not necessarily to work out the entire antigenic structure of the AcChR molecule but to generate a large number of probes for studying its structure and function. Atassi began his studies with a knowledge of both the amino acid sequence and the X-ray crystallographic structure of the proteins he studied. He found that each antigenic determinant comprised 6–7 amino acid residues. There are three determinants in lysozyme (of 129 total residues) and five in myoglobin (of 153 total residues). Four of the five determinants in myoglobin could be bound simultaneously by antibodies. The antigenic determinants were generally formed by exposed bends or the ends of segments

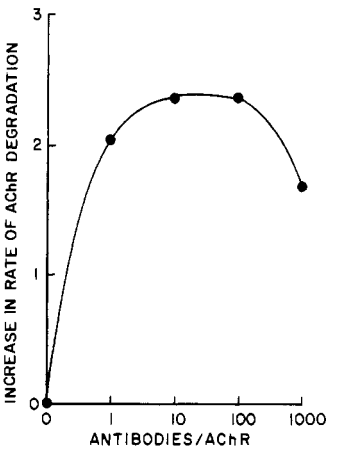

FIGURE 12: Dependence of AcChR destruction rate on concentration of mAb 35. AcChR destruction rate was measured as in Figure 10 in the continual presence of the indicated concentrations of mAb.

of polypeptide chain. Although determinants formed by linear sequences of amino acids depended strongly on the conformation of this polypeptide in the intact protein, cross-reaction was detectable with the denatured polypeptide fragment containing the binding site sequence. These were termed "continuous" binding sites. Some antigenic determinants are formed by amino acid residues which are close in the native protein, but separated in the sequence. These determinants are *absolutely* conformationally dependent. They were termed "discontinuous" binding sites.

From the observations of Atassi, we might expect one antigenic determinant for approximately every 37 amino acids in the AcChR monomer or approximately 70 total. Because part of each subunit would be occluded by other subunits or by membrane, one might expect fewer than 70. In the case of the α subunit, for example, one would expect something less than ten determinants. In fact, we can recognize at least six. These are the sites for binding mAbs: (1) 1 and 2, (2) 3 and 5, (3) 6 and 16, (4) 8, (5) 12, and (6) 14. The location on the surface of the AcChR monomer or the orientation of the polypeptide chain folds which form these determinants is such that, in all but the case of mAb 6 (mAb 16 was not tested), the two α subunits in a monomer can be cross-linked by a single antibody. Because the α subunit, and particularly the area to which mAb 6 binds, has a shape that makes it particularly immunogenic, nearly half of the antibodies in an anti-AcChR serum are directed at or near this point. This site is not unique to torpedo AcChR since mAbs 35 and 37 recognize a similar determinant on eel AcChR. Table I shows that seven of the 15 mAbs we investigated here are absolutely conformationally dependent and do not cross-react detectably with denatured AcChR polypeptide chains. Most or all of the antigenic determinants for these antibodies may be of the "discontinuous" type. The remaining 8 of these 15 mAbs react detectably, though usually not very well, with denatured subunits. The determinants for these antibodies are probably "continuous", formed by a linear sequence of amino acid residues. The actual size of the determinant on the AcChR molecule which enters the antibody binding site is probably much less than 20 Å, but the length of AcChR surface occluded from binding another mAb by the arm of the bound mAb might more nearly approximate the 30-Å diameter of an antibody arm.

The binding of mAb 6 to torpedo AcChR monomers at various antibody/AcChR ratios produced complexes of sizes consistent with those of an intermolecular cross-linking antibody (cf. Figure 1C). Because mAb 6 is known to react with

α, these results lend further support to the evidence that torpedo AcChR monomers contain two α subunits (Damle & Karlin, 1978; Lindstrom, et al., 1979a; Raftery et al., 1980). This antibody evidently cannot distinguish between the two α subunits, but they do differ in their ability to react with the affinity labeling reagent MBTA (Damle & Karlin, 1978) and in their ability to control the cation channel (Lindstrom et al., 1980a). mAbs 1, 2, 3, 5, 8, 12, and 14 produce complexes of the size of one antibody and one AcChR, independent of antibody/AcChR ratio. Thus, these antibodies must internally cross-link the two α subunits within an AcChR monomer (cf. Figure 1B). Evidently, they also cannot distinguish between the two α subunits. A mAb which reacted with only one of the two α subunits would produce both antibody cross-linked AcChR and complexes of one antibody and one AcChR, as shown in Figure 1A. A mAb which binds uniquely to the β, γ, or δ subunit would also be expected to behave in this way. mAb 7 presents a special case. It cross-reacts with both γ and δ and appears to cross-link these subunits within the monomer.

mAbs 35 and 37 react with the α subunit of eel AcChR at a site analogous to that recognized by mAb 6 on torpedo and cross-link eel AcChR monomers in the same pattern observed with mAb 6 and torpedo AcChR monomers. This indicates that eel AcChR also has two α subunits per monomer. This observation is consistent with the observations that eel AcChR contain four subunits comparable to the four subunits of torpedo AcChR (Lindstrom et al., 1980b), that the size of eel and torpedo AcChR monomers on sucrose gradients is identical, and that eel-like torpedo AcChR has two toxin binding sites and one MBTA binding site (Karlin et al., 1975).

mAb 35 was raised against eel AcChR but cross-reacts extensively with both torpedo and fetal calf muscle AcChR. It causes antigenic modulation of AcChR in fetal calf muscle cells. It is known that antigenic modulation is triggered by cross-linking of AcChR by antibody and is not caused by monovalent F(ab) (Drachman et al., 1978; Lindstrom & Einarson, 1979). It appears that mAb 35 cross-links AcChR in the plane of the membrane, forming complexes like those seen with soluble AcChR. This suggests that muscle AcChR also has two α subunits. This observation is consistent with the observations that muscle AcChR contains four sets of antigenic determinants comparable to the four subunits of torpedo AcChR (Lindstrom et al., 1978; 1979b), that the size of fetal calf muscle AcChR monomers is comparable to that of eel and torpedo, and that mAb 35 can intermolecularly cross-link muscle AcChR (Lindstrom et al., unpublished results; Lindstrom et al., 1981). The observation that the rate of modulation decreased at high antibody/AcChR ratios is consistent with the idea that under these conditions complexes of two antibodies and one AcChR are formed and that these, like F(ab)-labeled AcChR, are not destroyed at a more rapid rate. The increased rate of AcChR destruction is probably due to cross-linking of AcChR into aggregates which stimulate endocytosis. Aggregation of AcChR by antibody prior to endocytosis has been observed (Prives et al., 1979). Endocytosis appears to be the rate-limiting step in antigenic modulation, and, thereafter, the mechanism of AcChR destruction seems identical with that observed with normal AcChR (Lindstrom & Einarson, 1979).

It would be expected that internally cross-linking antibodies could not induce antigenic modulation because they cannot cross-link AcChR. Such antibodies are quite frequent and comprised seven of the ten antibodies we studied. Antibodies directed at a single determinant on a monomer such as β, γ, or δ could form aggregates no larger than two AcChR monomers (Figure 1A). Thus, it is problematic whether antibodies of this type alone could cause antigenic modulation. In the anti-AcChR sera of animals with EAMG and patients with MG, there is a mixture of antibody specificities which would no doubt be more effective at inducing modulation. However, it is clear that sera could differ in their ability to cross-link AcChR and induce modulation, depending on the population of antibody specificities in the serum and independent of its total anti-AcChR titer. Further, it is clear that the rate of antigenic modulation can show a biphasic dependence on antibody concentration and decrease in high antibody/AcChR ratios. Thus, it may be misleading to compare the rate of antigenic modulation with sera from different individuals at a single concentration chosen without consideration of cross-reacting antibody/muscle AcChR ratio.

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Effects of Thio-Group Modifications on the Ion Permeability Control and Ligand Binding Properties of *Torpedo californica* Acetylcholine Receptor†

Jeffery W. Walker, Ronald J. Lukas,‡ and Mark G. McNamee*

ABSTRACT: Chemical modification of membrane-bound *Torpedo californica* acetylcholine receptor by the disulfide reducing agent dithiothreitol has two major effects on receptor function: (1) it shifts the dose–response curve for agonist-induced increases in $^{22}Na^+$ permeability to 10-fold higher concentrations, and (2) it decreases the binding affinity of the receptor for the same agonist about 6-fold. In the experiments reported here, the agonist used was carbamoylcholine. Despite the quantitative changes in agonist binding and flux response, dithiothreitol-treated membranes display all other functional properties expected of the receptor. The flux response is blocked by preincubation of the membranes with carbamoylcholine, a phenomenon known as desensitization. In parallel, the receptor undergoes a carbamoylcholine-induced shift from a low-affinity to a high-affinity binding state for the same agonist. All of the effects of dithiothreitol are reversed by the oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid). Alkylation of the membranes with *N*-ethylmaleimide after dithiothreitol reduction results in complete inhibition of the flux response, and the effect is not reversed by the reoxidation treatment. The *N*-ethylmaleimide also shifts the receptor into a very low-affinity binding state for carbamylcholine that is shifted to only a slightly higher affinity by preincubation with carbamoylcholine. Prior to reduction, *N*-ethylmaleimide has no effect on receptor binding or flux properties. Detailed binding studies on affinity-alkylated receptor membranes indicate that the α-neurotoxin binding site not occupied by the affinity label displays all the same properties as unlabeled membranes, including the dithiothreitol and *N*-ethylmaleimide effects. The results are discussed in the context of several hypotheses previously proposed to account for the diverse effects of thio-group modifications on the acetylcholine receptor.

Understanding the relationship between the ligand binding and the ion permeability control properties of the nicotinic acetylcholine receptor (AcChR)[1] at postsynaptic membranes remains a major goal of current receptor research. One approach has been to characterize the effects of specific chemical modifications on the binding site and/or on the presumed ion channel. In one of the first chemical modification studies of a receptor, Karlin & Bartels (1966) showed that dithiothreitol (DTT) dramatically decreased the response of isolated electric eel electroplax to applied acetylcholine (AcCh). This result suggested the involvement of disulfides in normal AcChR function, since DTT was known to reduce disulfides to free sulfhydryls. The DTT effect was completely reversed by the oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid) [$(Nbs)_2$]. Alkylation of reduced electroplax with *N*-ethylmaleimide (MalNEt) prior to $(Nbs)_2$ treatment prevented reversal of the DDT effect (Karlin & Bartels, 1966).

DTT also altered the response of electroplax to other pharmacologic agents. For example, decamethonium, a bis-quaternary partial agonist, became a more potent agonist, and hexamethonium, normally an antagonist, became an activator of the reduced receptor (Karlin & Winnik, 1968). In addition, dose–response curves for carbamylcholine (Carb) showed a decreased affinity and a decreased slope of Hill plot (from 1.8 to 1.1), indicating a decrease in the apparent cooperativity of the response (Karlin, 1969).

Similar effects of sulfhydryl and disulfide modifications on physiologic responses have been measured on several vertebrate muscle preparations (Albuquerque et al., 1968; Mittag & Tormay, 1970; Rang & Ritter, 1971; Lindstrom et al., 1973; Ben Haim et al., 1973, 1975; Terrar, 1978) and on isolated neurones from the mollusc *Limnaea stagnalis* (Bregestovski et al., 1977). At the frog neuromuscular junction, DTT was shown to decrease both the lifetime and the conductance of single channels without any decrease in the number of functional channels (Ben Haim et al., 1975). In a recent study, bisulfite was found to enhance the depolarization response of the frog neuromuscular junction to AcCH (Steinacker, 1979). Unlike DTT, bisulfite heterolytically cleaves the disulfide to give a thiosulfate.

Karlin and co-workers also discovered [for reviews, see Karlin (1974, 1980) and Barrantes (1979)] that compounds containing both binding affinity for the AcChR and a sulfhydryl reactive group acted as affinity alkylating agents after

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[1] Abbreviations used: DTT, dithiothreitol; AcCh, acetylcholine; $(Nbs)_2$, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB in figures); AcChR, acetylcholine receptor; MalNEt, *N*-ethylmaleimide (NEM in figures); Carb, carbamoylcholine; BAC, bromoacetylcholine; MBTA, maleimidobenzyltrimethylammonium iodide; α-[^{125}I]BgTx, α-[^{125}I]bungarotoxin; PCMB, *p*-(chloromercuri)benzoate; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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