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Evidence for unpredicted transmembrane domains in acetylcholine receptor subunits

(monoclonal antibodies/synthetic peptide/transmembrane orientation/nicotinic)

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ABSTRACT **Two monoclonal antibodies (mAbs 236 and 237) against a synthetic peptide composed of the same amino acid residues as the sequence 152–167 of the α subunit of the acetylcholine receptor were obtained, and their crossreaction with the synthetic peptide, α subunit, and solubilized receptor was demonstrated. Crossreaction with the synthetic peptide α159–169 was less by a factor of 10^4, suggesting that the mAbs bind primarily to the sequence α152–159. Cholinergic ligands did not inhibit mAb binding. No crossreaction was observed with the receptor in native membranes, but the mAbs could bind to receptor reconstituted into liposomes in which 50% of the receptors have their cytoplasmic surface oriented outside. When native membranes were permeabilized with saponin, mAbs directed against cytoplasmic determinants of the receptor could bind to them, but mAbs 236 and 237 could not. However, after treatments that removed peripheral proteins from the cytoplasmic surface, binding of both mAbs was observed. Further evidence for the cytoplasmic localization of this sequence was provided by observation of partial competition for binding between mAbs 236 and 237 and mAbs previously demonstrated to bind to the cytoplasmic surface of the receptor. To account for these findings, a model for the organization of the polypeptide chains in receptor subunits is proposed that has a total of seven transmembrane domains in each subunit, two of which are amphipathic and one of which is not α-helical.**

The nicotinic acetylcholine receptor from fish electric organs and mammalian skeletal muscle is composed of four different homologous subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ (see reviews in ref. 1). Monoclonal antibodies (mAbs) have been extensively used in the study of the acetylcholine receptor, helping to identify receptor subunits; to localize substructures within subunits; to compare structures of receptors from different tissues and species; to study the synthesis, conformational maturation, and assembly of receptor subunits; and to study aspects of receptor function (reviewed in ref. 2). About one-half of the antibodies made to native receptors are directed at a small part of the extracellular surface of α subunits, termed the main immunogenic region (3–5). Most antibodies made to denatured receptor subunits are directed at the cytoplasmic surface of the receptor (6–8).

Analyses of the sequences of cDNAs for acetylcholine receptor subunits have led to several models for the transmembrane organization of the polypeptide chains in all of the subunits and the localization of particular domains, such as the acetylcholine binding site and main immunogenic region, on α subunits (9–13). Antibodies have proven useful in testing the predictions of such studies (14, 15). For example, the COOH termini of all subunits were found on the cytoplasmic surface, and the NH_2 termini of all subunits were found to be inaccessible to antibodies in the native receptor (14, 15), results consistent with the idea suggested by their sequence homologies that all of the subunits should have a fundamentally similar structure (10). Demonstration of the cytoplasmic localization of the COOH termini (14, 15, 47) proved that subunits could not have only the four hydrophobic α-helical transmembrane domains predicted in the models (9–11) but, instead, must have an odd number of transmembrane domains to account for an extracellular location of the NH_2 termini and a cytoplasmic location of the COOH termini. This result was consistent with two other models, which proposed a fifth amphipathic α-helical transmembrane domain (12, 13).

Noda *et al.* (16) proposed that the sequence α161–166 composed the main immunogenic region, because this sequence was highly hydrophilic. In fact, mAbs to the main immunogenic region do not bind to synthetic peptides containing this sequence (15, 17). Furthermore, we made mAbs to a synthetic peptide containing this sequence and showed that they did not compete for binding to native receptor with mAbs to the main immunogenic region (15). Here we demonstrate that these mAbs bind to the cytoplasmic surface of the receptor.

The observation that this part of the α subunit was located on the cytoplasmic surface was unanticipated by all previous models, all of which presumed that it was part of an extracellular domain extending from the NH_2 terminus to the first hydrophobic domain, which starts at about α210. Because this sequence is bracketed by two sequences that must be located on the extracellular surface, the only site for *N*-glycosylation (16) and a cysteine demonstrated to be adjacent to the acetylcholine binding site (18), demonstration of the cytoplasmic location of this sequence rather closely defines the boundaries of two previously unrecognized transmembrane domains. These domains are especially interesting because (*i*) one is too short to be α helical, (*ii*) the other is an amphipathic α-helix that contains many charged residues, as might be expected of a component of the cation channel through the membrane, and (*iii*) both domains at their extracellular surfaces have residues likely to be associated with the acetylcholine binding site, which might suggest that they are important in gating the opening of the cation channel.

Here we describe our evidence for the cytoplasmic location of the sequence α152–159 and discuss the implications of this observation.

MATERIALS AND METHODS

Antibody Preparation. The synthetic peptide Asp-Gly-Thr-Lys-Val-Ser-Ile-Ser-Pro-Gln-Ser-Asp-Arg-Pro-Asp-Leu, which corresponds to amino acids 152–167 of the α subunit, was synthesized and coupled to keyhole limpet hemocyanin by glutaraldehyde for use as an immunogen. Hybrid-

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Abbreviations: mAb, monoclonal antibody; MBTA, maleimidobenzyltrimethylammonium.

oma cell lines were obtained by fusing the mouse myeloma cell line S194 with spleen cells from a rat immunized with the peptide as described (3). Two hybridomas secreting mAbs 236 and 237 were obtained. Other antibodies directed against the receptor were obtained as described (3–5). The synthetic peptide Ser-Pro-Glu-Ser-Asp-Arg-Pro-Asp-Leu-Ser-Tyr, which corresponds to α159–169 plus a COOH-terminal tyrosine, was synthesized by Bachem (Torrance, CA).

ELISA. For screening the clones and investigation of antibody recognition, an ELISA was used (19). Briefly, receptor was bound to microtiter dishes (Immulon I, Dynatech, Alexandria, VA) by adding 50 μl per well of a 2×10^{-8} M solution in 0.01 M Na bicarbonate buffer (pH 9.5) overnight at 4°C. Addition of 200 μl of 0.5% bovine serum albumin/5% Tween 20/phosphate-buffered saline for 15 min to quench unreacted sites on the plates was followed by four washes with the same solution. In this way, binding of ligands subsequently used is prevented. mAbs were allowed to bind to the immobilized receptor for 4 hr at room temperature. mAb binding was measured using a mAb to rat IgG κ chains (MAR 18.5) (20) coupled to glucose oxidase (21) and sometimes mixed with goat anti-rat IgG coupled to the same enzyme.

An inhibition assay was designed to test the binding specificities of mAbs 236 and 237. Antibody (2 nM) was incubated at 20°C with different concentrations of synthetic peptides, α subunit (22), purified receptor (23), native and treated receptor-rich membranes (23), or receptor, reconstituted into vesicles composed of soybean lipids and cholesterol (4:1, wt/wt) (24). After 1 hr (in the case of soluble molecules) or overnight incubation (in the case of membranes), the amount of bound mAb was measured by ELISA. The same ELISA was used to test whether mAbs 236 and 237 competed for binding to the same region as other mAbs whose transmembrane orientation was known. For this purpose, each mAb of the pair of mAbs to be analyzed was incubated both individually and as a pair with immobilized receptor. Binding of the mAbs was detected using MAR 18.5 coupled to glucose oxidase. If both mAbs tested bound simultaneously, then an absorbance close to the sum of the absorbances obtained when the mAbs were incubated alone was obtained. On the contrary, if both mAbs competed for the same epitope, then only the absorbance due to the binding of one of them was obtained. This was quantitatively expressed with the additivity index of Friguet *et al.* (25).

RESULTS AND DISCUSSION

As shown in Fig. 1*A*, mAbs 236 and 237 bind to the synthetic peptide α152–167 against which they were raised. mAb 237 appears to have higher affinity for the peptide than does mAb 236. mAbs 236 and 237 bound with lower affinity (by a factor of 10^4) to a shorter synthetic peptide α159–169 (Ser-Pro-Glu-Ser-Asp-Arg-Pro-Asp-Leu-Ser-Tyr), which overlapped the COOH-terminal sequence of α152–167. This suggests that these mAbs bind amino acids in the sequence α152–159 and that their binding sites do not extend much beyond α159. mAb binding sites have been found to be composed of 6–8 amino acids (26). mAbs 236 and 237 bind with about the same affinity to purified α subunits (Fig. 1*B*) and solubilized receptors (Fig. 1*C*). On the other hand, no binding of mAbs 236 and 237 to native receptor-rich membranes was detected (Fig. 2) even with longer incubation times (18 hr). However, when receptor reconstituted into vesicles was used instead of native vesicles, mAb binding was observed (Fig. 2). No binding of antibodies to liposomes without receptor was detected (data not shown). The failure to observe binding of mAbs 236 and 237 to native vesicles, where the receptor is oriented almost exclusively right-side out (27), and evidence of mAb binding to reconstituted vesicles, where ≈50% of the receptor is located with its cytoplasmic

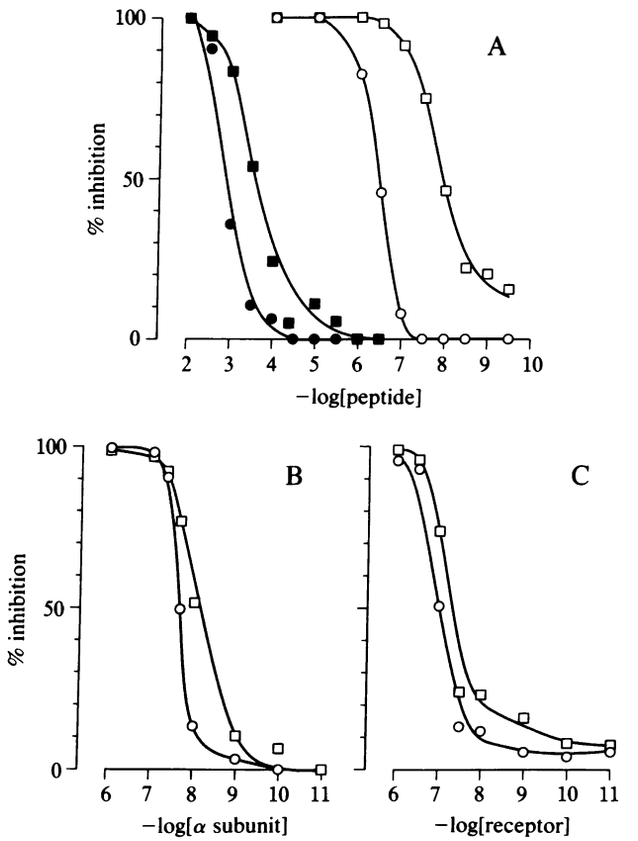

FIG. 1. Binding of mAbs 236 and 237 to synthetic peptides, α subunit, and soluble receptor. (*A*) Binding of mAbs 236 (○●) and 237 (□ ■) to synthetic peptides corresponding to residues α152–167 (open symbols) or α159–167 (closed symbols) was tested by the inhibition produced by the peptides on the binding of the mAbs to receptor bound to microtiter dishes. (*B*) Binding of mAbs 236 (○) and 237 (□) to α subunits under the same conditions as in *A*. (*C*) Binding of mAbs 236 (○) and 237 (□) to soluble receptor under same conditions as in *A* and *B*.

surface facing the outside (24), suggests that α152–159 is located on the cytoplasmic surface.

To further test the cytoplasmic localization of α152–159, two different approaches were followed. First, competition experiments between mAbs 236 and 237 and other mAbs directed against different parts of the receptor were carried out (Table 1). The cytoplasmic part of the receptor is a relatively small area in comparison with the extracellular portion (29). Therefore, if mAbs 236 and 237 bound to the cytoplasmic surface, one might expect some steric inhibition of the binding of mAbs 236 and 237 by some of the other mAbs that bind to the cytoplasmic face of the receptor. This was observed with mAbs 149, 111, 142, 151, and 118, which are specific for α or β subunits (28) and which bind to the cytoplasmic portion of the receptor as demonstrated by electron microscopy and colloidal gold labeling (6). This competition seems to be only partial and well differentiated (quantitatively) from the complete competition observed between mAbs 236 and 237 on one hand and the complete lack of competition with some other mAbs on the other hand (mAbs 125, 140, 6, 35, 203, 22, and 188).

The second approach to testing the cytoplasmic localization of α152–159 consisted of studying the binding of mAbs 236 and 237 to native membranes permeabilized with saponin or treated with lithium diiodosalicylate or alkaline pH to permeabilize and extract peripheral proteins (Table 2). Although saponin treatment was effective in permeabilizing the membrane and allowing the binding of several mAbs to the

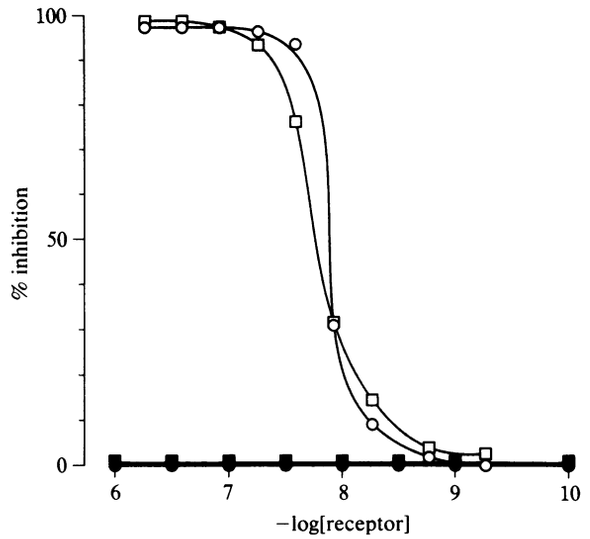

FIG. 2. Binding of mAbs 236 and 237 to receptor-rich native vesicles and receptor reconstituted into liposomes. Binding of mAbs 236 (○ ●) and 237 (□ ■) to native vesicles (closed symbols) or receptor reconstituted into vesicles composed of soybean lipids and cholesterol (open symbols) was tested as in the case of soluble molecules in Fig. 1. The indicated receptor concentrations refer to the total amount present in the membranes. In reconstituted vesicles, ≈50% of the receptor is located with its cytoplasmic surface facing the outside (24).

cytoplasmic part of the receptor, mAbs 236 and 237 could not bind. However, when the membranes were treated with alkaline pH or with lithium diiodosalicylate, significant binding of these mAbs was detected. Furthermore, if alkaline treatment was followed by saponin permeabilization, increased binding was observed. These data suggest that: (*i*) This sequence is located on the cytoplasmic face of the receptor, and (*ii*) peripheral protein extraction is necessary to expose the antigenic determinant. One possibility is that peripheral proteins bind to this sequence or close to it and only after their extraction can the mAbs bind. Another possibility, which does not exclude the first, is that after peripheral protein extraction, the receptor becomes more flexible, and the initially buried determinant is exposed. The possibility that lithium diiodosalicylate or pH 11 denatures the receptor cannot totally be ruled out; however, receptor function, which seems to be very sensitive to environmental changes (31, 32), is not affected by these treatments (30). Furthermore, two chemically unrelated treatments, lithium diiodosalicylate and alkaline pH, would not be expected to produce the same type of local modification if this were not related to their common target, peripheral protein extraction. Finally, the possible site(s) of interaction of peripheral proteins with the receptor could be located far away from this sequence and then the observed effect could simply be due to steric hindrance exerted by peripheral proteins. However, this does not seem to be the case, since several mAbs (149, 111, and 142), which appear to bind to adjacent sites in the receptor molecule because of their partial competition with mAbs 236 and 237, bind to the receptor without extraction of peripheral proteins.

Fig. 3 compares three models for the transmembrane orientation of the polypeptide chain in a receptor subunit and shows the model proposed by several groups (Fig. 3*A*) (9–11). This model proposes four transmembrane α-helical hydrophobic domains on the basis of analysis of the amino acid sequence of receptor subunits and proposes that α, β, γ, and δ subunits have the same basic structure because of the sequence homologies of the subunits (10). La Rochelle *et al.*

Table 1. Competition between mAbs 236 and 237, and mAbs directed against different antigenic determinants of the receptor

Competitor mAb	Subunit specificity and transmembrane orientation of antigenic determinant	Additivity index*	
		mAb236	mAb237
236	α	—	17
237	α	17	—
149	α, cytoplasmic†	43.2	41.8
111	β, cytoplasmic†	43.9	33.3
142	α,β,γ,δ, cytoplasmic†	54.5	48.3
151	β, cytoplasmic†	64.7	59.1
118	β, cytoplasmic†	56.0	42.9
125	β, cytoplasmic‡	100.0	81.0
127	δ, cytoplasmic‡	70.0	61.3
129	δ, cytoplasmic‡	78.3	72.6
131	δ, cytoplasmic‡	84.3	50.9
139	δ, cytoplasmic†	86.2	66.9
140	δ, cytoplasmic‡	92.7	71.6
6	α, extracellular MIR†	82.0	86.1
35	α, extracellular MIR†	96.0	91.4
203	α, extracellular MIR†	97.1	89.0
22	α, extracellular MIR†	82.7	92.3
188	α, extracellular MIR†	84.4	93.5

*Additivity index (25) is expressed as follows: AI = $100[(2A_{1+2}/A_1 + A_2) - 1]$ where A_1 is the absorbance with mAbs 236 or 237 alone, A_2 is the absorbance of the competitor mAb alone, and A_{1+2} is the absorbance with the two mAbs together. A low value of the AI indicates that the two mAbs cannot bind simultaneously and compete for binding, whereas a value near 100 indicates that both mAbs can bind to receptor simultaneously and do not compete for binding. Intermediate values may indicate partial competition.

†Transmembrane orientation of mAb binding determined by electron microscopy (6). MIR, main immunogenic region.

‡Transmembrane orientation of mAb binding determined by proteolytic cleavage and immunoprecipitation (28).

Table 2. Reaction of mAbs with native and treated receptor-rich membranes

mAb	Antigen localization	% inhibition of binding of mAb to immobilized receptor				
		Native vesicles	Saponin-treated*	LIS-treated*	pH 11-treated*	pH 11- and saponin-treated*
236	α	7.0	21.3	63.1	71.2	93.5
237	α	11.3	26.4	73.0	77.0	95.9
203	α, extracellular MIR	94.6	98.9	92.5	—	—
149	α, cytoplasmic	17.5	97.1	92.0	84.7	—
111	β, cytoplasmic	21.3	94.0	89.7	85.5	—
154	γ, cytoplasmic	11.5	98.8	90.4	—	—
139	δ, cytoplasmic	9.1	96.9	84.6	67.7	—
142	α,β,γ,δ, cytoplasmic	19.1	100.0	94.5	88.2	—

*Native receptor-rich membranes were permeabilized with 0.3% saponin (7) or extracted with pH 11 (30) or 10 mM lithium diiodosalycilate (LIS) (7). In all cases receptor in membranes was present at 1 μM.

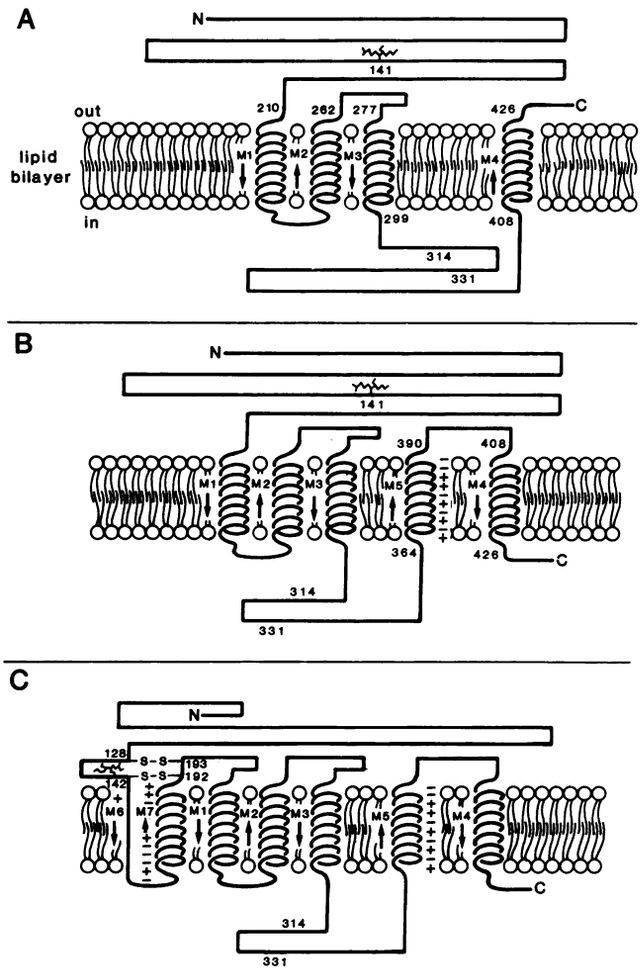

FIG. 3. Models of polypeptide chain organization in acetylcholine receptor subunits. (*A*) The four hydrophobic transmembranous domain model proposed by Claudio *et al.* (9), Noda *et al.* (10), and Devillers-Thiery *et al.* (11) on the basis of an analysis of the distribution of hydrophobic residues in the sequences of receptor subunits. (*B*) A model with a fifth amphipathic transmembrane domain proposed by Finer-Moore and Stroud (13) and Guy (12) as a result of a different theoretical analysis. (*C*) A model with two additional amphipathic domains to account for the transmembrane orientation of various sequences determined by immunochemical and other techniques. Sequence numbering is for α subunits (16). The organization of the polypeptide chain in the extramembranous domains is not specifically depicted, except to suggest that the NH_2 terminus is not exposed on the subunit surface (13, 33). Carbohydrate is shown attached at α141 (16) and a double disulfide bond is shown between α128 or 142 and α192 or 193 (18). It is proposed that the α subunits differ in glycosylation (34–36). This may affect which disulfide occurs with α192 or α193, which may in turn account for the observation (37) that one α is easily reduced and labeled with MBTA, while the other is difficult to sustain reduced and reactive with MBTA.

(38) have reported that mAbs to the sequence γ360–377 bind to the cytoplasmic surface, which is consistent with this model and the other models shown. In this model, both the NH_2 terminus and the COOH terminus of each subunit are on the extracellular surface. Previously, we showed that the number of transmembrane domains predicted by this model could not be correct because the COOH termini of α, β, γ, and δ were located on the cytoplasmic surface (14, 15). This feature, along with the accessibility and cytoplasmic location of all the COOH termini (14, 15, 47), is consistent with the argument (10) that all of the subunits should have homologous structures.

Fig. 3*B* shows the model proposed by Finer-Moore and Stroud (13) and Guy (12) on the basis of analysis of the subunit's amino acid sequences by another computerized technique. This model proposes a fifth amphipathic helix and is consistent with our COOH-terminal orientation data (14, 15) and that of R. Stroud and co-workers (47). The amphipathic helix proposed has all of its charged residues on one side, and it is appealing to think that this side of the helix from each subunit might form the barrel stave (39), which is believed to be contributed by each subunit to the lining of the cation channel through the center of the molecule. Neither model A nor model B is consistent with the data we present in this paper, which indicates that the α subunit sequence 152–159 is exposed on the cytoplasmic surface.

Model C adds two transmembrane domains to model B to account for our observation. This model has several specific features. It is known that α subunits are cotranslationally glycosylated at a single site (40, 41). The only site at which *N*-glycosidic linkage would be expected is asparagine-141 (16). Therefore, model C proposes that α141 is on the extracellular surface. However, we do not have direct evidence that glycosylation occurs at α141. If glycosylation does occur at α141, this only leaves 12 amino acids to α152, which we find on or close to the cytoplasmic surface. Twelve amino acids could form a chain long enough to extend across the ≈32-Å hydrophobic core of the membrane in an extended conformation (43 Å, at 3.6 Å per residue) but not in an α-helix (only 18 Å, at 1.5 Å per residue) (42). Transmembrane domains need not be α-helical. For example, porin contains primarily β structure and little α-helix (42, 43). Kao *et al.* (18) have shown that the cysteine at α192 is labeled by the acetylcholine binding site affinity ligand maleimidobenzyltrimethylammonium (MBTA). Therefore, this residue must be on the extracellular surface. There are 31 amino acids between α159, which we know to be on or near the cytoplasmic surface, and α192, which we know to be on the extracellular surface. This is sufficient to form an α-helix across the membrane. It is interesting that Finer-Moore and Stroud (13) predict that part of this region forms an α-helix with all of its charged residues on one side, just as in the amphipathic helix, which they propose forms part of the lining of the channel. The two closely adjacent transmembrane domains that we propose form a loop, which is likely to place the binding site cysteine residues α192α193 adjacent to the cysteine residue α142. Kao *et al.* (18) and Luyten *et al.* (44) have proposed that α142 is disulfide linked to α192 or α193 because it is known that α192 and α193 are involved in disulfide bonds, but there is no known example of adjacent cysteines being disulfide bonded. In addition, if the sequence α152–159 were close to the binding site, as previously proposed by model B (13), one should expect at least some steric inhibition between α-bungarotoxin and mAbs 236 and 237, given the size of these macromolecules. However, we have observed that α-bungarotoxin and mAbs 236 and 237 do not compete for binding to the receptor. Furthermore, it is interesting that the boundary between exon P5, which encodes α96 to α160 (45), and exon P6, which encodes α161 to α240, comes at the boundary between the proposed first transmembrane domain M6 and the second transmembrane domain M7. This might be expected if exons correspond to structural domains in the proteins they encode. This model predicts that adjacent residues, asparagine-α141 and cysteine-α142, are involved in glycosylation and the acetylcholine binding site. This is consistent with several observations that suggest the two α subunits in a receptor differ in glycosylation near the cysteine that reacts with MBTA. Hall *et al.* (34) showed that a monospecific antibody from a patient with myasthenia gravis bound near to one of the two acetylcholine binding sites. We (35) and Conti-Tronconi *et al.* (36) showed that the two α subunits differ in glycosylation, which could account for their differential reaction with MBTA (37). Since model C

proposes three amphipathic transmembrane domains (M5, M6, and M7), it is possible that they could self-associate on the extracellular surface after they are synthesized and then coordinately insert across the membrane before the association of α with the other subunits. In this respect, it is interesting that a conformation change in α subunits has been identified that occurs before their association with the other subunits and that affects the area of the acetylcholine binding site (46). The present model has been built from data obtained for a very specific sequence of the α subunit and bearing in mind several features very specific for the α subunit too, so that it can only be rigorously applied to this subunit. However, given the homologies found between subunits, it seems reasonable to assume the same structure for every subunit of the receptor.

Many more experiments will be required to test and refine the proposed model. For example, current data on the transmembrane orientation of α141 (16), α152–159, and α192–193 (18) tightly constrain the existence and orientation of the transmembrane domains M6 and M7. The cytoplasmic exposure of the COOH terminus of each subunit (14, 15) requires that each subunit have an odd number of transmembrane domains. However, there is no experimental evidence yet for the existence or orientation of the domains M1–M5 proposed according to theoretical analysis of subunit amino acid sequences (9–13). We have included all of those domains in model C because this results in an odd number of transmembrane domains. Further experiments using monoclonal antibodies to synthetic peptides and other techniques will be required to establish which of these subunit sequences in fact forms transmembrane domains.

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