Transmembrane Topography of Nicotinic Acetylcholine Receptor: Immunochemical Tests Contradict Theoretical Predictions Based on Hydrophobicity Profiles†

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ABSTRACT: In our preceding paper [Ratnam, M., Sargent, P. B., Sarin, V., Fox, J. L., Le Nguyen, D., Rivier, J., Criado, M., & Lindstrom, J. (1986) *Biochemistry* (preceding paper in this issue)], we presented results from peptide mapping studies of purified subunits of the *Torpedo* acetylcholine receptor which suggested that the sequence β429–441 is on the cytoplasmic surface of the receptor. Since this finding contradicts earlier theoretical models of the transmembrane structure of the receptor, which placed this sequence of the β subunit on the extracellular surface, we investigated the location of the corresponding sequence (389–408) and adjacent sequences of the α subunit by a more direct approach. We synthesized peptides including the sequences α330–346, α349–364, α360–378, α379–385, and α389–408 and shorter parts of these peptides. These peptides corresponded to a highly immunogenic region, and by using ^{125}I-labeled peptides as antigens, we were able to detect in our library of monoclonal antibodies to α subunits between two and six which bound specifically to each of these peptides, except α389–408. We obtained antibodies specific for α389–408 both from antisera against the denatured α subunit and from antisera made against the peptide. These antibodies were specific to α389–396. In binding assays, antibodies specific for all of these five peptides bound to receptor-rich membrane vesicles only after permeabilization of the vesicles to permit access of the antibodies to the cytoplasmic surface of the receptors, suggesting that the receptor sequences which bound these antibodies were located on the intracellular side of the membrane. Electron microscopy using colloidal gold to visualize the bound antibodies was used to conclusively demonstrate that all of these sequences are exposed on the cytoplasmic surface of the receptor. These results, along with our previous demonstration that the C-terminal 10 amino acids of each subunit are exposed on the cytoplasmic surface, show that the hydrophobic domain M4 (α409–426), previously predicted from hydropathy profiles to be transmembranous, does not, in fact, cross the membrane. Further, these results show that the putative amphipathic transmembrane domain M5 (α364–399) also does not cross the membrane. Our results thus indicate that the transmembrane topology of a membrane protein cannot be deduced strictly from the hydropathy profile of its primary amino acid sequence. We present a model for the transmembrane orientation of receptor subunit polypeptide chains which is consistent with current data.

Antibodies against synthetic peptides are reliable probes for studying protein structure as they have frequently been found to cross-react specifically with the native proteins containing these sequences (Niman et al., 1983). Antibodies against synthetic peptides corresponding to various parts of the primary sequence of the nicotinic acetylcholine receptor have been used to visualize the binding domains of these antibodies in receptor-rich membranes (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Criado et al., 1985a,b; Young et al., 1985; La Rochelle et al., 1985). The rationale for choosing the synthetic peptides in these studies was based on plausible theoretical models of the receptor structure deduced from the hydrophobicity profiles of the primary sequences of the subunits of the receptor (Noda et al., 1983; Claudio et al., 1983; Devillers-Thiery et al., 1983; Guy, 1983; Finer-Moore & Stroud, 1984).

Various aspects of the structure of the receptor that have been studied so far, and the theoretical models proposed for the transmembrane orientation of its subunit polypeptide chains, were discussed in the introduction of our previous paper (Ratnam et al., 1986). As described in that report, we mapped the binding sites of several monoclonal antibodies (mAbs)[1] on the primary structure of α, β, and δ subunits of the receptor by peptide mapping. These studies led to the surprising conclusion that a hydrophobic sequence (M4) which several groups (Noda et al., 1983; Claudio et al., 1983; Devillers-Thiery et al., 1983; Guy, 1983; Finer-Moore & Stroud, 1984) proposed to be a transmembrane domain in receptor subunits did not span the membrane in the β subunit. We arrived at

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[1] Abbreviations: Boc, *N-tert*-butoxycarbonyl; BOP, benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DOB, dichlorobenzyl; Ig, immunoglobulin; LIS, lithium diiodosalicylate; mAb, monoclonal antibody; MIR, main immunogenic region; PBS, phosphate-buffered saline [10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl]; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; EtDT, ethanedithiol; TEAP, triethylammonium phosphate; Da, dalton(s).

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this conclusion because the site recognized by mAb 125, which binds to the cytoplasmic side of the receptor, could be mapped to the immediate N-terminal side of M4 on the β-subunit sequence and because the C-terminus of the subunit is also cytoplasmic (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985). In this report, we extend this conclusion to the α subunit by using antibodies to a synthetic peptide (α389–408) from the α-subunit sequence which corresponds to the mAb 125 binding site on β subunits. Ratnam et al. (1986) also showed that a number of mAbs in our library bound to the region α333–385 on the α-subunit sequence on peptide maps. Here we have synthesized short peptides covering this sequence and used them to map precisely the sequences which bind these mAbs. Then we used several techniques to demonstrate that these mAbs bind to the cytoplasmic surface of the receptor.

The locations on the cytoplasmic surface of several sequences of the α subunit have previously been demonstrated by using mAbs (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Criado et al., 1985a,b), and two additional sites are known to be exposed on the extracellular surface of α subunits, namely, the site of affinity labeling by a cholinergic analogue (Kao et al., 1984) and the site of cotranslational N-glycosylation (Noda et al., 1982; Mishina et al., 1985). We present a model for the transmembrane orientation of the α-subunit chain which incorporates these data as well as the data obtained in this paper and which indicates aspects of the transmembrane domain structure which are still uncertain. Our studies indicate that the transmembrane topology of the receptor subunits cannot be predicted strictly on the basis of hydropathy profiles.

Materials and Methods

Solubilized Receptor and Membrane Vesicles. Receptor was purified from the electric organs of *Torpedo californica* by affinity chromatography on toxin agarose (Lindstrom et al., 1981a). Receptor-rich membrane vesicles were prepared from the same source by sucrose density gradient centrifugation (Elliot et al., 1980). These vesicles were permeabilized to antibody molecules by hypotonic shock for electron microscopy or by pH 11.2 (Neubig et al., 1979) to more thoroughly disrupt closed vesicle structures for other experiments involving quantitative radioimmunoassays. Vesicles were also permeabilized by treatment with lithium diiodosalicylate (LIS) or saponin (Froehner et al., 1983).

Peptides. Peptides corresponding to the sequences α330–346, α349–364, α360–378, α379–385, and α389–408, and shorter parts of these peptides, were synthesized. All the peptides had an additional tyrosine residue at the C-terminus to permit radioiodination and also coupling to carrier protein for immunization.

All *N-tert*-butoxycarbonyl (Boc) amino acids were of the L configuration and purchased from BACHEM (Torrance, CA), with the exception of di-Boc-His, which was prepared and coupled without previous neutralization according to procedures described in the literature (Le Nguyen et al., 1985). Manual solid-phase peptide synthesis was used to prepare the protected peptide resins (Barany & Merrifield, 1980). Chloromethylated resin Bio-Beads SX-1 (Bio-Rad, 200–400 mesh, 1.25 mequiv of Cl/g) were esterified (Gisin, 1973) with Boc-Tyr (dichlorobenzyl) to a substitution of 0.2 and 0.5 mmol/g. The coupling steps were performed with the use of benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Le Nguyen et al., 1985). This reagent provided a coupling via hydroxybenzotriazole active esters, allowing most of the reactions to be completed within 30 min.

Each coupling cycle was performed as follows. The *deprotection* cycle involved the following steps: (1) treat Boc-peptide resins with trifluoroacetic acid (TFA) 50% in dichloromethane (DCM) containing 1% ethanedithiol (EtDT) for 1 min; (2) repeat operation 1 for 30 min; (3) wash with 1% EtDT in 2-propanol (1 time); (4) wash with DCM (3 times). The deprotected peptide resin is then ready for the next coupling step without neutralization. *Coupling* consisted of the following procedures: (1) addition of BOP (0.6 mmol/g) and Boc-amino acid (0.6 mmol/g); (2) addition of solvent (DCM) [for Boc-Trp and Boc-Asn (xanthenyl), the mixture of dimethylformamide in DCM (50:50 v/v) was used]; (3) addition of diisopropylethylamine until pH 9 (~9 equiv); (4) after 10 min, a resin sample was collected for ninhydrin testing, the pH was controlled by using pH indicator sticks (if the pH was below 7, more diisopropylethylamine was added), and the reactions were usually completed within 30 min; (5) MeOH wash (twice); (6) DCM wash (twice).

Amino acids were incorporated in the following protected forms: Boc-Tyr (2,6-dichlorobenzyl), di-Boc-His (dicyclohexylamine salt), Boc-Asp (*O*-benzyl), Boc-Lys (2-chlorobenzyloxycarbonyl), Boc-Glu (*O*-benzyl), Boc-Asn (xanthenyl), Boc-Ser (benzyl). Tryptophan and methionine were incorporated with unprotected side chains.

The peptide was cleaved from the resin by HF. Anisole (1.5 mL) and dimethyl sulfide (0.5 mL) were added per gram of peptide resin. HF (ca. 10 mL/g of resin) was added to this suspension cooled in liquid nitrogen. The reaction mixture was allowed to warm to 0 °C in 0.5 h followed by another 0.5–0.75-h incubation with stirring. HF was evaporated under vacuum, the resin/peptide was washed with ether, and the peptides were extracted in dilute acetic acid and lyophilized.

Purification was performed by using preparative HPLC under conditions previously described (Rivier et al., 1984).

Characterization of the peptides by optical rotation and HPLC behavior in two systems is reported in Table I. Analytical HPLC was performed on a Vydac C_{18} column using two buffer systems: TEAP/CH_3CN, A = triethylammonium phosphate buffer, B = 60% CH_3CN in A; 0.1% TFA/CH_3CN, A = 0.1% TFA in H_2O, B = 60% CH_3CN in A. Amino acid analyses after 4 N methanesulfonic acid hydrolysis (110 °C for 24 h under vacuum in sealed ampules) gave the expected values.

Iodinations. α-Bungarotoxin and peptides were labeled with ^{125}I using chloramine T to specific activities of 5×10^{17} to 2×10^{18} cpm/mol (Lindstrom et al., 1981a).

Peptide Coupling and Immunization. Peptide (Tyr-409) α389–409 (3.6 mg) was coupled to the carrier protein keyhole limpet hemocyanin (4.8 mg) by using bisdiazotized benzidine (Bassiri & Utiger, 1979). Residual uncoupled peptide was not removed before immunization. Three young female Lewis rats were injected with two doses of 100 μg of peptide per rat (intradermally and intraperitonially) in Freund's complete adjuvant with an interval of 2 weeks. The rats were bled after 4 weeks.

Solution Assays of Antibodies. Antisera were assayed by reacting with ^{125}I-α-bungarotoxin-labeled receptor (1 nM) in 100 μL of 10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl/0.5% Triton X-100/10 mM NaN_3, precipitating the immune complex with 100 μL of goat anti-rat immunoglobulin, diluting with 1 mL of buffer, pelleting, washing the precipitate with 2 × 1 mL of the buffer, and counting the radioactivity, all as described earlier (Lindstrom et al., 1981a). The binding of mAbs to ^{125}I-peptides was assayed similarly by using 5 μM ^{125}I-peptide and various dilutions of mAbs in 100-μL volumes.

Table I: Characterization of Synthetic Fragments of the α Subunit of Acetylcholine Receptor

subunit fragments (sequence)	$[\alpha]^{20}_D$[a]	RT(A)[b]	gradient condn[c]	RT(B)[b]	gradient condn[c]
[Tyr-347]α330–347 (KRASKEKQENKIFADDIY)	−43.6	6.8	25–40 (15)	11.1	25–40 (20)
[Tyr-347]α339–347 (NKIFADDIY)	−43.7	9.0	25–45 (20)	12.2	25–45 (20)
[Tyr-365]α349–365 (SDISGKQVTGEVIFQTY)	−24.8*	8.0	30–45 (15)	11.5	28–48 (20)
[Tyr-365]α357–365 (TGEVIFQTY)	−18.4*	6.1	30–45 (15)	11.6	25–45 (20)
[Tyr-379]α360–379 (VIFQTPLIKNPDVKSAIEGY)	−89.7	11.9	30–50 (20)	8.5	35–55 (20)
[Tyr-379]α370–379 (PDVKSAIEGY)	−56.8	7.1	20–40 (20)	9.1	20–40 (20)
[Tyr-386]α371–386 (DVKSAIEGVKYIAEHY)	−21.3	11.2	35–55 (20)	9.3	40–55 (15)
[Tyr-386]α379–386 (VKYIAEHY)	−20.4*	8.1	14–34 (20)	14.1	14–34 (20)
[Tyr-409]α389–409 (DEESSNAAEEWKYVAMVIDHY)	−14.2	10.3	45–60 (15)	10.5	45–60 (15)
[Tyr-409]α396–409 (AEEWKYVAMVIDHY)	−20.3*	8.4	42–52 (10)	6.5	45–60 (15)
[Tyr-409]α403–409 (AMVIDHY)	−38.7	8.8	17–27 (10)	10.2	20–35 (15)

[a] Measured in 50% acetic acid; concentration = 1 or 0.5 (represented by asterisk). [b] Retention times (RT) are reported in minutes. (A) and (B) are systems A and B described under Materials and Methods. [c] Gradient conditions indicate % B in each system, e.g., from 25% to 40% in 15 min (numbers in parentheses are minutes).

Competition assays for the binding of anti-peptide antibodies were done in 10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl (PBS) using 1–10 nM ^{125}I-peptide and 0.4 μL of an anti-α-subunit serum, 0.1–0.5 μL of anti-peptide serum, or a 1–5 nM sample of an appropriate mAb per 100 μL. The binding of ^{125}I-peptide to antibody was assayed in triplicate in the presence of various concentrations of solubilized receptor, native receptor vesicles, or vesicles treated with pH 11.2, LIS, or saponin overnight at 4 °C. Peptide complexed with antibody was precipitated with goat anti-rat immunoglobulin (100 μL); after dilution with 1 mL of buffer and pelleting, the precipitate was washed with 2 × 1 mL of PBS and the radioactivity counted. These values were expressed as the percent of the maximal cpm, i.e., in the absence of receptor.

Competition between anti-peptide antibodies and other mAbs that bound to receptor-rich membranes was determined in the following way. ^{125}I-Peptide and anti-peptide antibodies were incubated in PBS (100 μL) with pH 11.2 treated vesicles alone or with the vesicles preincubated (overnight at 4 °C) with various mAbs, and the ^{125}I-peptide–antibody complex was precipitated and counted as described above. If a mAb competed with the anti-peptide antibodies for binding to receptor in the vesicles, then it reduced significantly or prevented the inhibition of the binding of anti-peptide antibodies to ^{125}I-peptide by alkaline-extracted vesicles.

Electron Microscopy. The transmembrane orientation of epitopes recognized by specific mAbs was determined by the technique of Wray and Sealock (1984) and La Rochelle et al. (1985). This technique was learned during a visit by P.B.S. to Dr. Robert Sealock's laboratory at The University of North Carolina. Receptor-rich membranes were purified by the method of Elliott et al. (1980), centrifuged to the bottom of poly(vinyl chloride) assay wells, and lysed by treatment with distilled H_2O for 5 min. Membranes were typically exposed to two antibody "sandwiches" in succession (La Rochelle et al., 1985). The first sandwich was constructed by incubating membranes successively with an mAb of unknown transmembrane orientation (titer of 0.2–0.5 μM), with rabbit anti-rat Ig (0.2 μM, Cappell), and with 6-nm colloidal gold–protein A. The second sandwich was constructed by incubation with a "reference" mAb of known transmembrane orientation, with rabbit anti-rat Ig, and with 15-nm gold–protein A. The orientation of reference mAbs 35 (extracellular) and 111 (cytoplasmic) was established by comparing their pattern of binding to that of *Naja naja siamensis* toxin, which is known to bind to the extracellular domain of the receptor. The transmembrane orientation of any reactive mAb can, in theory, be deduced by simply noting the distribution of small gold particles associated with receptor-rich membranes in relation to the large gold labeling produced by incubation of membranes with either extracellular or intracellular reference mAbs. This approach is similar to that employed by La Rochelle et al. (1985), who used cobra toxin in the second sandwich as a reference probe.

Colloidal gold was made according to the technique of Muhlpfordt (1982, 6-nm gold) or Slot and Geuze (1981, 15-nm gold) and was conjugated to protein A as described by Slot and Geuze (1981) and purified as described by Wray and Sealock (1984).

Results

Immunogenicity and Antigenicity of Peptide [Tyr-409]-α389–409. An antiserum against the denatured α subunit cross-reacted about 10% with the peptide ^{125}I-[Tyr-409]-α389–409, indicating that this sequence is highly immunogenic in the denatured α subunit. The sequence specificity of the cross-reacting antibodies in the anti-α-subunit serum was tested further by examining the binding of the antibodies to ^{125}I-[Tyr-409]α389–409 in the presence of various concentrations of the unlabled peptides, [Tyr-409]α403–409, [Tyr-409]-α396–409, and [Tyr-409]α389–409. As can be seen in Figure 1A, only the unlabeled peptide [Tyr]α389–408 could inhibit the binding of antibodies to the iodinated peptide, indicating that the antigenic determinant is within the sequence α389–396.

The peptide was also quite immunogenic, producing anti-peptide titers of 0.6–0.8 μM in the sera of immunized rats. As with the anti-α-subunit serum, the anti-peptide serum was also specific for the N-terminal half of the peptide, as indicated by the finding that only unlabeled [Tyr-409]α389–409 and not unlabeled [Tyr-409]α403–409 or [Tyr-409]α396–409 could compete for the binding of ^{125}I-[Tyr-409]α389–409 to these antibodies (Figure 1B). The immunogenicity of the amino-terminal part of [Tyr-409]α389–409 was further confirmed by the observation that rats immunized with peptide [Tyr-409]α396–409 showed no detectable anti-peptide titers in their sera.

Antisera against [Tyr-409]α389–409 cross-reacted about 6–10% with intact detergent-solubilized receptor in solution radioimmunoassay, indicating that some of the anti-peptide antibodies can bind to the conformation of this sequence present in the native receptor. That this cross-reaction did not result from a denatured fraction of the receptor but that this sequence is actually exposed in the native receptor is indicated by the fact that in antibody excess all the ^{125}I-α-bungarotoxin-labeled receptor in a reaction mix could be immunoprecipitated.

Competition Assays for the Binding of Antibodies to [Tyr-409]α389–409 to the Receptor in Membranes. The binding of antibodies from antisera to α subunits that cross-

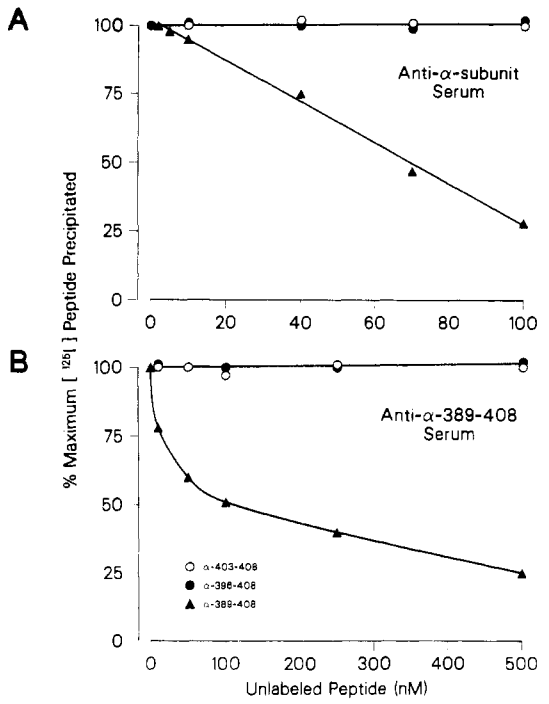

FIGURE 1: Competition for antibodies against ^{125}I-[Tyr-409]α389–409 using unlabeled peptides. Antiserum to the α subunit (A) or antiserum to the peptide [Tyr-409]α389–409 (B) was incubated with ^{125}I-[Tyr-409]α389–408 (1 nM), and the immune complex was precipitated with goat anti-rat immunoglobulin and counted for radioactivity. The precipitation of the ^{125}I-peptide was measured in the presence of various concentrations of unlabeled [Tyr-409]α389–409 (▲), [Tyr-409]-α396–409 (●), or [Tyr-409]α403–409 (○) and was expressed as percent of control, i.e., precipitation in the absence of unlabeled peptides.

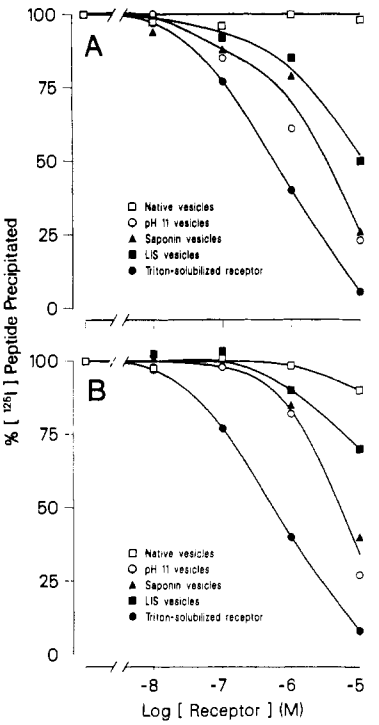

FIGURE 2: Inhibition of the binding of antibodies to ^{125}I-[Tyr-409]α389–409 by receptor in various forms. ^{125}I-[Tyr-409]α389–409 (1 nM) was incubated with an antiserum against the denatured α subunit (A) or with antiserum against the peptide (B) in the absence and in the presence of various concentrations of native receptor-rich vesicles (□), pH 11.2 treated vesicles (○), saponin- treated vesicles (▲), LIS-treated vesicles (■), or Triton X-100 solubilized receptor (●). The ^{125}I-peptide that could be precipitated with goat anti-rat immunoglobulin was expressed as percent of cpm precipitated in the absence of receptor.

react with peptide [Tyr-409]α389–409 to detergent-solubilized receptor and to the receptor in membranes is shown in Figure 2A. In this experiment, we used native membrane vesicles in which the receptors are oriented "outside out" in the membrane as well as the vesicles permeabilized to the antibodies by treatment with pH 11.2, saponin, or LIS. Immunoprecipitation of a low concentration of the iodinated peptide by a similar low concentration of cross-reacting antibodies was inhibited by high concentrations of solubilized receptor or permeabilized receptor vesicles, but not by native membrane vesicles (Figure 2A). This result suggests that the sequence α389–396 is on the intracellular side of the receptor, since the antibodies had access to it only when the membranes were treated in ways known to permeabilize them.

Figure 2B shows the binding of antibodies in antiserum to [Tyr-409]α389–409 to the iodinated peptide, assayed in the presence of various concentrations of solubilized receptor, native receptor vesicles, or the vesicles permeabilized by pH 11.2, saponin, or LIS treatment. The results with this anti-peptide serum were similar to those obtained above with the antiserum to α subunits; i.e., native receptor vesicles did not bind efficiently to the anti-peptide antibodies, whereas solubilized receptor and permeabilized vesicles did bind to them. This suggests that this sequence is on the cytoplasmic side of the membrane.

Competition between Antibodies to the Sequence [Tyr-409]α389–409 and Other Anti-Receptor Antibodies. The results from the preceding experiments could also be explained if the sequence α389–396 were in fact extracellular but only made accessible to antibodies by local denaturation caused by treatment with pH 11.2 or detergents. Therefore, a more direct way to determine which side of the membrane this sequence occurs on was to look for competition between the anti-peptide antibodies and mAbs whose sidedness of binding was established directly by electron microscopy (Sargent et al., 1984; this study). mAbs binding to the same side of the membrane, especially to the α subunit, might compete at least partially with these antibodies for binding to receptor in the membrane, given the large size (~150000 Da) of an antibody molecule. The mAbs chosen for this experiment were three antibodies (mAbs 35, 6, and 210) against the main immunogenic region (MIR) of the receptor, which is known to be on the extracellular side of the α subunit (Tzartos & Lindstrom, 1980; Tzartos et al., 1981; Sargent et al., 1984), and also three anti-α-subunit antibodies (mAbs 142, 149, and 187), an anti-β-subunit antibody (mAb 111), an anti-γ-subunit antibody (mAb 132), and an anti-δ-subunit antibody (mAb 139), all of which bind to the intracellular surface of the receptor (Sargent et al., 1984; Anderson et al., 1983).

In this experiment, a concentration of receptor in pH 11.2 treated vesicles (2 μM) was chosen that could inhibit, by more than half, the amount of ^{125}I-[Tyr-409]α389–409 that could be immunoprecipitated with the antiserum to α subunits. The inhibition of binding of ^{125}I-peptide to the anti-peptide antibodies was assayed both in the presence of pH 11.2 treated vesicles alone and also in the presence of the vesicles that had been preincubated overnight with a high concentration (~6 μM) of various mAbs. As can be seen in Figure 3, none of the anti-MIR antibodies had any effect on the inhibition by pH 11.2 treated vesicles. On the other hand, mAbs 142, 149, 187, and 111 afforded significant protection against this inhibition (Figure 3). Further, when these antibodies were used in combination (mAbs 142 + 149 and mAbs 187 + 111) with each of the mAbs being present at only half the earlier con-

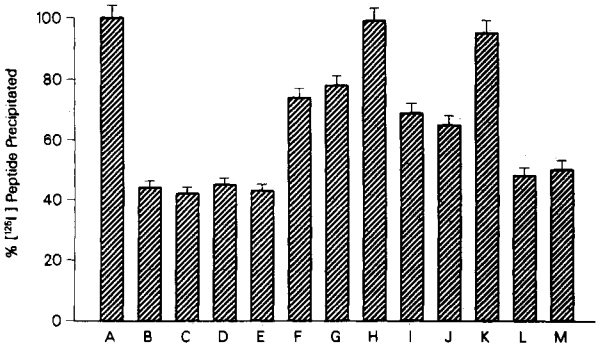

FIGURE 3: Competition between antibodies to [Tyr-409]α389–409 and various mAbs for binding to the receptor in pH 11.2 treated membrane vesicles. The binding of ^{125}I-[Tyr-409]α389–409 to cross-reacting antibodies in an antiserum to the α subunit was assayed by precipitating the peptide–antibody complex with goat anti-rat immunoglobulin in the absence of receptor (A), in the presence of pH 11.2 treated vesicles alone (B), or in the presence of vesicles preincubated with mAb 35 (C), mAb 210 (D), mAb 6 (E), mAb 142 (F), mAb 149 (G), mAbs 142 + 149 (H), mAb 187 (I), mAb 111 (J), mAbs 187 + 111 (K), mAb 132 (L), and mAb 139 (M). The amount of ^{125}I-peptide precipitated in the absence of receptor was taken as 100%.

centration (3 μM), they afforded almost complete protection against inhibition by pH 11.2 treated vesicles, showing that their effects were due to partial blocking of the binding of the anti-peptide antibodies to the receptor. These results suggest that antibodies against the sequence α389–396 bind to the cytoplasmic side of the receptor.

Cross-Reaction of Monoclonal Antibodies with Synthetic Peptides. Evidence that α389–396 is on the cytoplasmic surface of the receptor contradicts the models of Guy (1983) and Finer-Moore and Stroud (1984), which predicted that this sequence was on the extracellular surface between the putative amphipathic transmembrane domain M5 and the putative hydrophobic transmembrane domain M4. This sequence was predicted to be on the cytoplasmic side of the receptor in models having four hydrophobic transmembrane domains, M1–M4 (Claudio et al., 1983; Noda et al., 1983a; Devilliers-Thiery et al., 1983). However, there is now strong evidence that the C-terminus (α428–437) is located on the cytoplasmic surface (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985; Ratnam et al., 1986). Taken together, the data that both α389–396 and α428–437 are on the cytoplasmic surface suggest that M4 (α408–426) is also in the cytoplasmic part of the receptor and is not a transmembrane domain. These data also question the existence of M5. Therefore, we investigated this region. From peptide mapping studies, we knew that this region was highly immunogenic (Ratnam et al., 1986). Consequently, we synthesized peptides covering the sequence α330–386 and used them to screen our library of anti-α mAbs for mAbs which could be used to determine the transmembrane orientation of this region.

mAbs to α subunits in our library were screened by solution radioimmunoassays for binding to synthetic ^{125}I-labeled peptides covering the sequence 330–408 of the α subunit (Table II). A number of mAbs cross-reacted strongly with peptides in the region α330–378. The pattern of binding of these mAbs to the peptides is shown schematically in Figure 4.

Transmembrane Orientation of the Binding Sites of Mapped mAbs. As shown in Figure 4, the mAbs that bound to the various synthetic peptides in the region α330–378 could be divided into groups having the same sequence specificity. To locate these sequences on one side of the membrane or the other, the binding of one mAb from each group was studied. The binding of these mAbs to corresponding ^{125}I-labeled

Table II: Cross-Reaction of mAbs with Synthetic Peptides of the α Subunit[a]

	titer (nM) vs. peptide										
mAb	[Tyr-347]-α330–347	[Tyr-347]-α339–347	[Tyr-365]-α349–365	[Tyr-365]-α357–365	[Tyr-379]-α360–379	[Tyr-379]-α370–379	[Tyr-386]-α371–386	[Tyr-386]-α378–389	[Tyr-409]-α389–409	[Tyr-409]-α396–409	[Tyr-409]-α403–409	deduced mAb binding site[b]
149	15.2	13.9	0	0	0	0	0	0	0	0	0	α339–346
187	22.8	28.7	0	0	0	0	0	0	0	0	0	α339–346
3	0	0	51.3	0	0	0	0	0	0	0	0	α349–357
5	0	0	44.6	0	0	0	0	0	0	0	0	α349–357
142	0	0	3820	21.0	0	0	0	0	0	0	0	~α353–359
8	0	0	0	0	10.5	4.0	0	0	0	0	0	~α366–372
10	0	0	0	0	1.43	0	0	0	0	0	0	α360–370
147	0	0	0	0	4760	0	0	0	0	0	0	α360–370
61	0	0	0	0	0	0	3.82	0	0	0	0	α371–378
152	0	0	0	0	0	0	741	0	0	0	0	α371–378
153	0	0	0	0	0	0	2490	0	0	0	0	α371–378
155	0	0	0	0	0	0	6330	0	0	0	0	α371–378
157	0	0	0	0	0	0	4020	0	0	0	0	α371–378
164	0	0	0	0	0	0	220	0	0	0	0	α371–378

[a] Note that all of these mAbs failed to bind at 0.5 background with the other peptides tested. This strongly indicates the specificity of the binding which was observed. Further, none of the mAbs to the M1R and several other mAbs bound to any of the peptides tested. The other peptides tested were [Tyr-11]α1–11, [Tyr-100]α100–116, α127–143, [Tyr-170]α159–170, [Tyr-171]α171–189, [Tyr-193]α193–212, α261–277, and [Tyr-427]α427–437. Titer means moles of peptide bound per liter of antibody solution. Minimum detectable titers are less than 0.1 nM. [b] Where an antibody site overlapped two peptides or was limited by their overlap, the limits of its size were chosen by assuming that six amino acids formed a site.

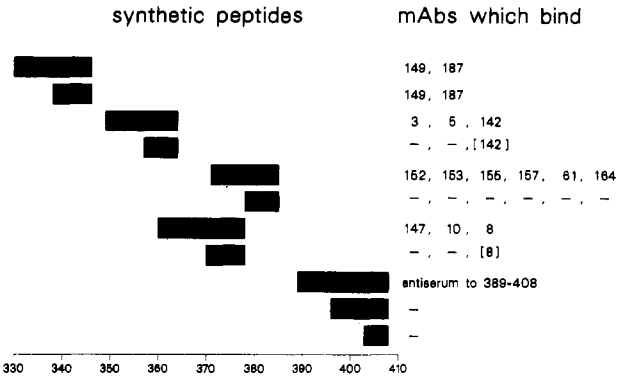

FIGURE 4: Sequence specificities of antibodies that bind in the region α330–408 on the primary sequence of the α subunit. Various short synthetic peptides are represented by horizontal lines. The positions of these peptides on the primary sequence of the α subunit are indicated by the long line at the bottom. Antibodies that bind to each of the longest synthetic peptides or shorter fragments thereof are indicated in a column on the right of corresponding peptides. Brackets denote mAbs that cross-react with reduced affinity with a shorter peptide as compared to the larger peptide.

peptides was examined in the absence and in the presence of various concentrations of native receptor vesicles, of vesicles permeabilized by treatment at pH 11.2, or of detergent-solubilized receptor (Figure 5). The binding of all of these mAbs to the peptides was inhibited effectively by permeabilized vesicles or by solubilized receptor, while much higher concentrations (nearly 100-fold) of receptor in native vesicles were needed to produce the same amount of inhibition, suggesting that these mAbs bound on the cytoplasmic side of the membrane. The inhibition at high concentrations of native vesicles is expected since a small percentage of these vesicles are known to be sealed "inside out". It can be seen in Figure 5 that less receptor is needed to inhibit the binding of these mAbs to peptides than is needed to inhibit the binding of antibodies against the peptide [Tyr-409]α389–409 (Figure 2). This difference is explained by the difference in relative affinities of these antibodies for peptide and receptor. The antisera used in Figure 2 react better with the peptides, whereas the mAbs used in Figure 5 have been selected for their high affinity for receptor.

Figure 5 also shows mutual competition among mAbs of the four groups which bind to sequences between α339 and α378 for binding to the receptor in alkali-treated membranes. In this case, the inhibition of binding of mAb 157 to ^{125}I-labeled peptide [Tyr-386]α378–386 by pH 11.2 treated vesicles was examined in the absence and in the presence of the other mAbs. These mAbs protected against the inhibition significantly when used singly, and even better when used in combination with other protecting mAbs, indicating that they competed with mAb 157 and with one another for binding to the membrane-bound receptor. This further suggests that the sequences recognized by all of these antibodies are located on the same side of the membrane.

mAb binding to lysed receptor-rich vesicles was visualized in the electron microscope after incubation of lysed vesicles with a sandwich consisting of a mAb, rabbit anti-rat Ig, and small (6 nm) colloidal gold–protein A. The transmembrane orientation of mAb binding was determined by means of a second sandwich consisting of a reference mAb of known orientation, rabbit anti-rat Ig, and large (15 nm) colloidal gold–protein A. The reference mAbs consisted of mAb 35, which binds to the extracellular domain of the receptor, and mAb 111, which binds to the intracellular domain (Sargent et al., 1984). In a preliminary experiment, the binding of these

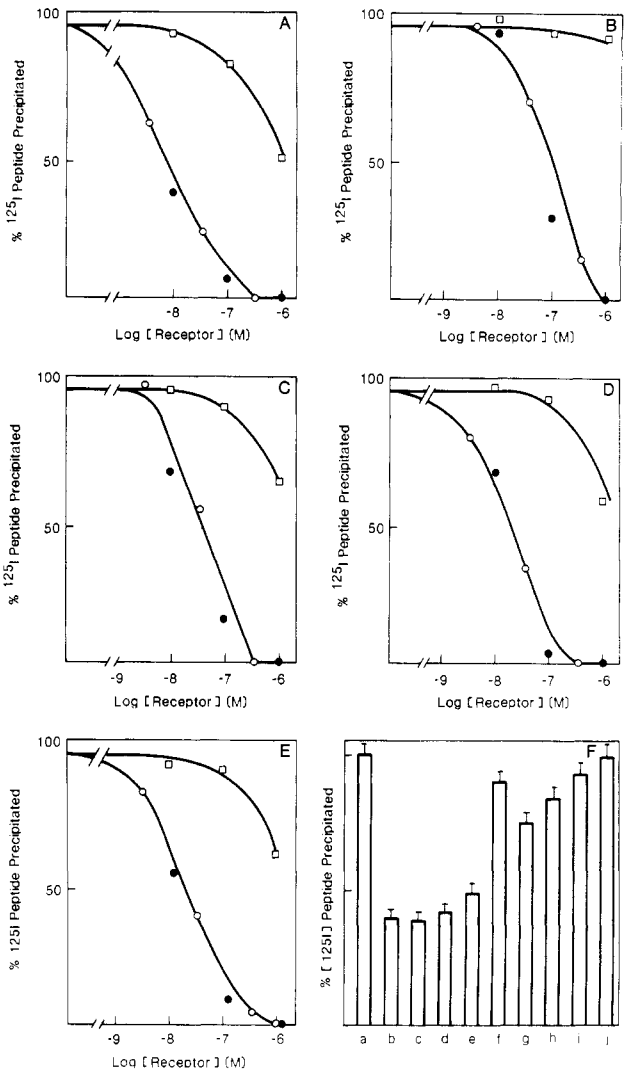

FIGURE 5: Binding of mAbs to alkali-treated receptor vesicles. Various ^{125}I-labeled peptides covering the sequence α330–385, i.e., [Tyr-365]α349–365 (A), [Tyr-347]α330–347 (B and C), [Tyr-386]-α371–386 (D), and [Tyr-379]α360–379 (E) at concentrations of 1–10 nM, were incubated with mAbs 142 (A), 149 (B), 187 (C), 157 (D), and 147 (E) at concentrations of 2–5 nM in the absence and in the presence of various concentrations of native receptor vesicles (□), alkali-treated vesicles (○), or Triton X-100 solubilized receptor (●). The ^{125}I-peptide that could be precipitated with goat anti-rat immunoglobulin was expressed as percent of cpm precipitated in the absence of receptor. In panel F, the ^{125}I-labeled peptide [Tyr-386]α371–386 (10 nM) was incubated with mAb 157 (1 nM) in the absence (a) and in the presence of alkali-treated receptor vesicles (30 nM) alone (b) or with vesicles preincubated with 100 nM mAbs 6 (c), 210 (d), 142 (e), 149 (f), 187 (g), 147 (h), 142 plus 149 (i), and 147 plus 187 (j). The ^{125}I-peptide that could be precipitated in each case with goat anti-rat immunoglobulin is plotted in panel F as percent of the total cpm precipitated in the absence of receptor (a).

two mAbs was compared to that of *Naja naja siamensis* toxin in order to ensure that their binding to receptors in lysed vesicles was well-behaved. Toxin competes for acetylcholine binding and thus recognizes the extracellular domain of the receptor. Figure 6 shows the binding of cobra toxin alone and when preceded by mAbs 35 and 111. The large gold particles are indicative of toxin binding and mark the extracellular side of receptor-rich membranes. Clearly, mAb 35 binds to the same side of the membrane as toxin, the outside, and mAb 111 binds to the opposite side of the membrane as toxin, the inside.

Of the 14 mAbs that cross-reacted with peptides in the region α330–378 (Table II), 8 were found to bind detectably

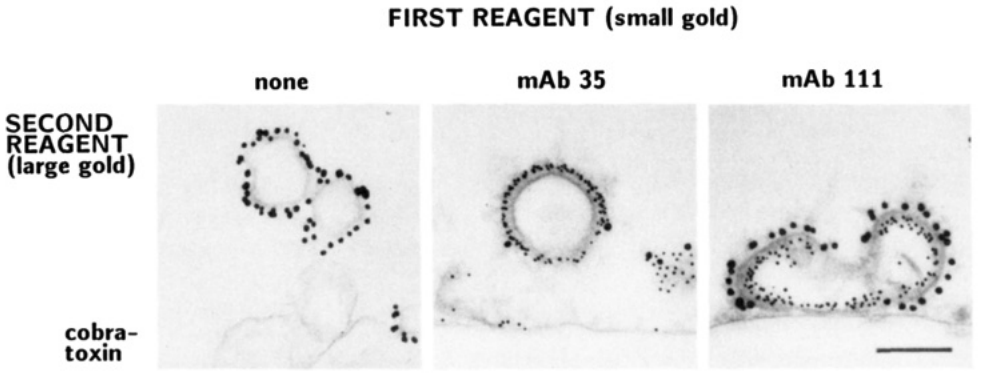

FIGURE 6: Location of mAb and cobra toxin binding sites on receptor-rich membranes. Membranes immobilized on the bottom of poly(vinyl chloride) assay wells were treated with cobra toxin, with rabbit anti-rat Ig, and with 15-nm colloidal gold–protein A as described by Wray and Sealock (1984) and La Rochelle et al. (1985). For the middle and right micrographs, membranes were preincubated with the mAb indicated, with rabbit anti-rat Ig, and with 6-nm colloidal gold–protein A. The results demonstrate that mAb 35 binds to vesicles on the same side as cobra toxin and that mAb 111 binds to vesicles on the opposite side as cobra toxin. Since cobra toxin binds to the extracellular surface of the receptor, mAb 35 must recognize an extracellular epitope, and mAb 111 must recognize an intracellular one. Bar (in right-hand micrograph), 200 nm.

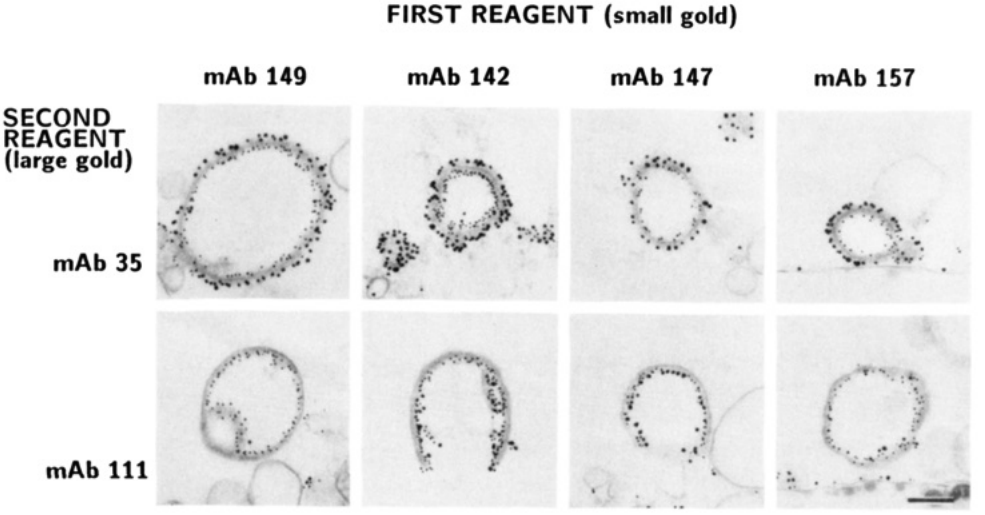

FIGURE 7: Visualization of mAb binding to receptor-rich membranes. Each column of two micrographs represents results from a double sandwich incubation consisting of an experimental mAb, rabbit anti-rat Ig, 6-nm colloidal gold–protein A, a reference mAb (35 or 111), rabbit anti-rat Ig, and 15-nm colloidal gold–protein A. Each of the four mAbs illustrated binds on the opposite side of the membrane as mAb 35 and, thus, is recognizing a cytoplasmic epitope. The density of large gold particles is typically weak when these mAbs are followed by mAb 111, due probably to steric hindrance (see, however, mAb 147). Large gold particles found outside the layer of small gold probably do not represent mAb–receptor binding but rather the recognition of exposed protein A molecules of the first sandwich by antibody molecules of the second sandwich. Bar (in lower right-hand micrograph), 200 nm.

to receptor-rich vesicles. These mAbs included at least one antibody specific for each of the following peptides: α339–346 (mAbs 149 and 187), α 349–357 (mAb 5), α353–359 (mAb 142), α360–370 (mAb 147), and α371–378 (mAbs 153, 155, and 157). All eight reactive mAbs were found to bind to the inside of the membrane, as illustrated for four of the mAbs in Figure 7. In each instance, small gold particles, indicative of mAb binding, are found on the side of the membrane *opposite* that recognized by mAb 35 (Figure 7, upper row). Relatively little binding of mAb 111 was observed following incubation of membranes with these mAbs, presumably because of steric hindrance (Figure 7, lower row). These results nicely complement those obtained by immunochemical means and strongly suggest that the sequence α339–α378 is on the intracellular surface of the receptor.

Discussion

The present study was initiated due to evidence from peptide mapping data of the receptor subunits (Ratnam et al., 1986) that the binding site of an antibody localized on the cytoplasmic side of the receptor could be mapped to the sequence 429–441 on the β subunit. This sequence was proposed to be extracellular in some theoretical models of the receptor structure (Guy, 1983; Finer-Moore & Stroud, 1984) and intracellular in other models (Claudio et al., 1983; Noda et al., 1983a; Develliers-Thiery et al., 1983). Therefore, further investigations were undertaken to identify the location of the sequence 389–408 on the α subunit, as this sequence corresponds to the position of β429–441 upon aligning homologous segments of the α- and β-subunit sequences (Noda et al., 1983a). For this purpose, the peptide [Tyr-409]α389–409 was synthesized. Antibodies against this peptide were obtained both from a cross-reacting anti-α-subunit serum (this sequence was apparently highly immunogenic in the denatured α subunit) and also from rat antisera made against the peptide. These antibodies reacted exclusively with the sequence α389–396 (Figures 1 and 4). Our studies with these antibodies showed that the sequence α389–396 is in the intracellular portion of the receptor, as receptors oriented outside out in membrane vesicles could bind the antibodies only when permeabilized (Figures 2 and 3). This conclusion was confirmed by the competition observed between the anti-peptide anti-

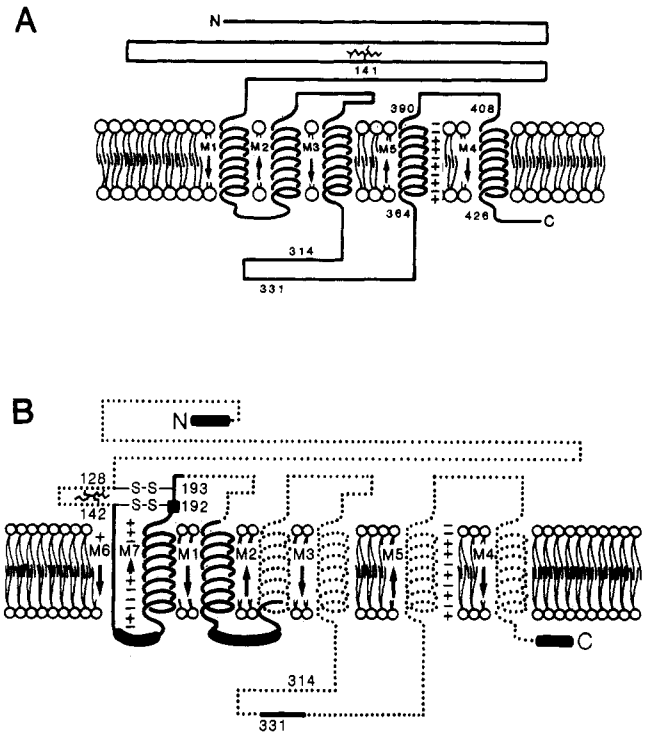

FIGURE 8: Diagrammatic representation of models for the transmembrane structure of the α subunit of the receptor. (A) Theoretical model proposed by Finer-Moore and Stroud (1984). (B) Modification of model A by Criado et al. (1985b). The dark lines in model B indicate sequences whose locations have been experimentally determined, whereas the dotted lines indicate sequences whose transmembrane orientation was uncertain at the time this model was proposed.

bodies and mAbs known to bind to the cytoplasmic side of the membrane (Figures 3 and 5).

As expected from the peptide mapping studies reported in the preceding paper (Ratnam et al., 1986), a number of mAbs in our library cross-reacted with peptides in the region α330–378. The transmembrane orientation of the binding sites of these mAbs was determined. All these antibodies bound to the receptor-rich vesicles only when the vesicles were permeabilized and, further, competed with one another for binding to the receptor in membranes (Figure 5), suggesting that these mAbs bound on the cytoplasmic surface of the receptor. Their binding to the internal side of the membrane in receptor-rich vesicles was clearly visualized by electron microscopy (Figure 7).

Current models of acetylcholine receptor structure are expected to fulfill the following minimal criteria: (1) hydrophobic and hydrophilic segments of the protein should be in hydrophobic and hydrophilic environments, respectively; (2) the central cation channel should have polar amino acid residues in its lining, since the receptor channel behaves electrophysiologically as a water-filled pore (Huang et al., 1978; Lewis & Stevens, 1979; Lewis, 1979; Horn & Stevens, 1980); (3) the protein mass distribution across the membrane should be consistent with data obtained by electron microscopy and X-ray diffraction techniques, according to which about 2–3 times the total protein mass is present on the extracellular side as compared with the portion of the receptor on the cytoplasmic side (Brisson & Unwin, 1985). While current theoretical models can adequately fulfill the above criteria, experimental evidence in recent studies (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985; Criado et al., 1985a,b) contradicts some of these models, and evidence reported here contradicts all of these models.

Figure 8A depicts a two-dimensional model of the structure of an individual subunit of the receptor in the membrane as proposed by Finer-Moore and Stroud (1984). This model was modified recently to accommodate two additional transmembrane domains (M6 and M7) as shown in Figure 8B (Criado et al., 1985a). M6 is a hydrophilic sequence, and M7 is an amphipathic α helix. The existence of M6 and M7 was suggested by evidence that mAbs specific for the sequence α152–159 bound to the cytoplasmic surface (Criado et al., 1985a). The sequence α235–242 was similarly assigned to the cytoplasmic side of the receptor by using two mAbs specific for this sequence (Figure 8B) (Criado et al., 1985b). Interestingly, antibodies against the sequences α152–159 (mAb 236), α235–242 (mAb 255), and α339–346 (mAb 149) did not compete with each other for binding to denatured α subunits but did compete with each other for binding to the native receptor, indicating that these sequences, which are distant in the primary sequence of α, are close to each other in the native receptor (Criado et al., 1985b). The regions in the primary sequence of the α subunit that previously have been located by using antibodies against synthetic peptides having these sequences are indicated by dark lines in Figure 8B. These are (1) the C-terminus of the subunit (α428–437) (Ratnam & Lindstrom, 1984; Lindstrom et al., 1984; Young et al., 1985; Ratnam et al., 1986), (2) α152–159 (Criado et al., 1985a), and (3) α235–242 (Criado et al., 1985b). In addition, there is evidence that the N-terminus is extracellular (Anderson et al., 1982). However, the N-terminus was found to be inaccessible to antibodies in the native receptor conformation (Ratnam & Lindstrom, 1984; Neumann et al., 1984). The fact that affinity alkylating reagents label cysteines α192 and α193 (Kao et al., 1984) shows that these residues are extracellular. Further, since asparagine-141 is the only potential site for the observed N-glycosylation on the α subunit (Merlie et al., 1982; Anderson & Blobel, 1981; Mishina et al., 1985; Noda et al., 1982), this residue should also be extracellular.

Using immunochemical techniques, we have located the sequence α389–396 on the cytoplasmic surface of the receptor. This sequence is between the putative transmembrane domains M5 and M4 (Figure 8A). Since the C-terminus (α428–437) is also on the cytoplasmic surface (Ratnam & Lindstrom, 1984; Ratnam et al., 1986), our evidence indicates that the hydrophobic α helix, M4, is not a membrane-spanning sequence. Further, we have located the sequences α339–346, α353–359, α360–370, and α371–378 on the cytoplasmic surface of the receptor as well. These sequences extend from about 40 residues from the C-terminal side of M3 (Figure 8B) and cover most of the putative amphipathic channel-forming domain, M5. Thus, M5 is a cytoplasmic domain because it is accessible to antibodies only from the cytoplasmic surface. Our results therefore provide strong evidence that the α subunit between residue 339 and the C-terminus is entirely on the cytoplasmic side of the membrane-bound receptor. These findings are illustrated in Figure 9.

The existence of hydrophobic α helixes outside the membrane could be explained if they formed the core of globular domains of the protein, as is seen in many globular proteins (Dill, 1985). Likewise, hydrophilic amino acid residues could exist in the membrane if they were packed in a suitable environment in the three-dimensional structure of the protein, e.g., as seen in bacteriorhodopsin (Engelman et al., 1982). The presence of M5 on the cytoplasmic side raises the question of mass distribution (Brisson & Unwin, 1985). The most recent estimate of the ratio of the extracellular volume to the in-

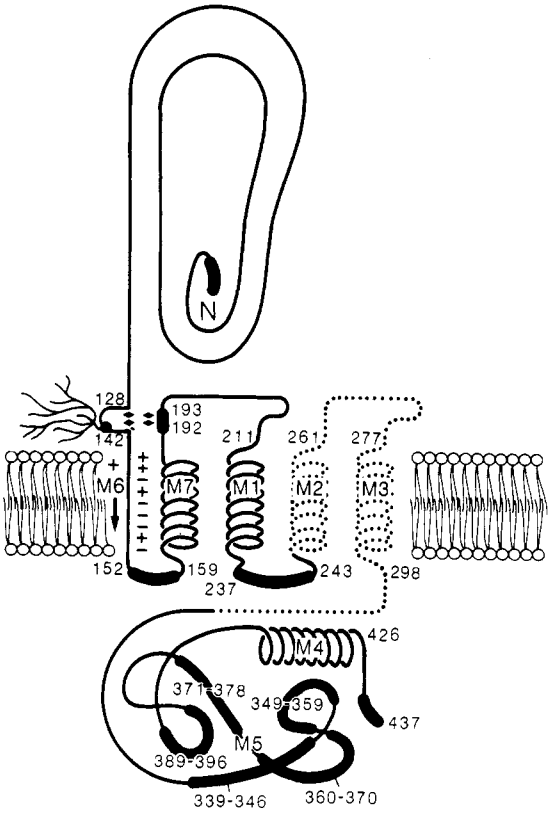

FIGURE 9: Model of the transmembrane orientation of the polypeptide chain in α subunits.

tracellular volume of the receptor by analysis of electron micrographs of negatively stained preparations is 2–3 (Brisson & Unwin, 1985). The model in Figure 8 has a ratio for amino acids on the extracellular surface to the cytoplasmic surface of about 1.25. Carbohydrate equivalent to about 3–7% of the mass of the receptor (Lindstrom et al., 1979; Vandlen et al., 1979) on the extracellular surface would increase this ratio to around 1.4. If M4 were associated with the hydrophobic core of the membrane, but not transmembranous, the ratio would increase to 1.8. Sufficient uncertainties remain both in the measurements of mass distribution and in the transmembrane orientation of parts of the polypeptide chain to warrant no special concern over this point.

Recently, Mishina et al. (1985) used the approach of site-directed mutagenesis to determine the importance of various parts of the receptor for its functional integrity. They produced deletions in each of the putative membrane-spanning regions (M1–M5). They found that deleting M1, M2, M3, M4, or small segments of M5 resulted in loss of receptor function and suggested that these regions might, therefore, represent transmembrane domains. We argue that deletions in any part of a protein could affect its function indirectly by producing a change in overall conformation and/or by altering subunit assembly. On the other hand, it was found (Mishina et al., 1985) that deletion of the sequence α355–389, which includes the whole M5, resulted in partially functional receptors. This is consistent with the idea that M5 is not the lining of a transmembrane channel as had been proposed, but is entirely cytoplasmic, as we have shown.

Domains of the receptor from *Torpedo californica* and *Torpedo marmorata* in contact with lipid were investigated by using photoreactive arylazidophospholipids (Giraudat et al., 1985). It was found that an 8000-dalton C-terminal peptide of the α subunit was labeled at the surface of the lipid bilayer in both species. Since the labeling was 5-fold higher in the case of *T. marmorata* as compared with *T. californica*, the labeled site was presumed to be the only mutation site in this region of the α subunit in the two species, i.e., at residue α424, which is a cysteine in *T. marmorata* and a serine in *T. californica*. This residue falls within the limits of M4 on the α subunit, and, therefore, M4 was suggested to be in contact with lipid (Giraudat et al., 1985). Our results, while strongly suggested that M4 is not transmembranous, do not preclude the possibility that M4 is partially embedded in the lipid.

The observed binding of mAb 10 to the sequence α360–370 is interesting because this antibody was reported to noncompetitively block channel activity (Lindstrom et al., 1981b; Blatt et al., 1985). Since this effect of mAb 10 was observed only when it was introduced on the extracellular surface of the receptor (Lindstrom et al., 1981b; Blatt et al., 1985), the binding site of this mAb was assumed to be extracellular. In this study, however, we found strong evidence for the cytoplasmic location of the sequence α360–370 (e.g., by electron microscopy, mAb 147, which binds to this sequence, was shown to bind to the cytoplasmic surface). An explanation for this apparent discrepancy could be that mAb 10 also binds to some other sequence on the external side of the receptor which mediates its functional effect. This suggestion is supported by the fact that mAb 10 binds with a much lower affinity to peptides, to denatured α subunit, or to intact receptor than does mAb 147 or any other mAb mapped by us in this report (Table I). In fact, the antibody binds better to the β subunit (Tzartos & Lindstrom, 1980), which shows no obvious sequence homology with the sequence α360–370 (Noda et al., 1983). mAb 10 could, thus, recognize some features of the peptide α360–370 which occur in other amino acid sequences as well.

Here we provide evidence that neither a proposed hydrophobic domain (M4) nor a proposed amphipathic transmembrane domain (M5) exists. Previously, we have provided evidence for two other transmembrane domains, both near the acetylcholine binding site, one short and perhaps strained (M6), as might perhaps be appropriate to a domain involved in channel opening, and the adjacent one amphipathic (M7), as might be appropriate for lining a hydrophilic channel (Criado et al., 1985a).

Figure 9 is a model which summarizes our current knowledge of the transmembrane orientation of the polypeptide chain in α subunits. The line depicts the polypeptide backbone on which the positions of some residues are indicated. Thick-line segments indicate sequences whose transmembrane orientation has been explicitly determined by the presence of carbohydrate in the case of α141 (Mishina et al., 1985), by affinity labeling with an acetylcholine analogue in the case of α192, 193 (Kao et al., 1984), and by the binding of mapped mAbs in all other cases (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Ratnam et al., 1986; Criado et al., 1985a,b; Young et al., 1985; La Rochelle et al., 1985; this paper). Thin-line segments indicate sequences whose transmembrane orientation seems very likely due to constraints placed by the location of surrounding sequences. Dotted-line segments indicate sequences whose transmembrane orientation has yet to be demonstrated. M1–M4 were proposed to be hydrophobic, α-helical transmembrane domains by Claudio et al. (1983), Noda et al. (1983a), and Devillers-Thiery et al. (1983). These models had the C-terminus on the extracellular surface. However, there is now evidence that the C-terminus of all the subunits is on the cytoplasmic surface (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985; Ratnam et al., 1986). Here we show that sequences bracketing M4 are

on the cytoplasmic surface; thus, the sequence α396–428 containing M4 must traverse the membrane either twice or, more likely, not at all. Instead of this hydrophobic sequence crossing the membrane, it is more likely to be in peripheral contact with lipid or in the core of a globular domain. M5 was proposed to be an amphipathic, α-helical transmembrane domain by Guy (1983) and Finer-Moore and Stroud (1984) and to correspond to approximately α342–378. The existence of an odd number of transmembrane domains would be consistent with the cytoplasmic location of the C-terminus, and an amphipathic helix could contribute to a hydrophilic lining for a cation channel through the center of the receptor. However, here we explicitly show that this region is in the cytoplasmic domain. All models have proposed that the sequence between M1 and M2 was exposed on the cytoplasmic surface, and Criado et al. (1985b) provided immunochemical evidence that α235–242 is, in fact, on the cytoplasmic surface. Since Kao et al. (1984) have localized α192 on the extracellular surface near the acetylcholine binding site, it is likely that M1 is, in fact, a transmembrane domain between α192 on the outside and α235–242 on the inside. Noda et al. (1982) proposed that α161–166 was the most hydrophilic sequence on the extracellular surface and formed the MIR. However, Lindstrom et al. (1984) showed that this was not the MIR, and Criado et al. (1985a) showed that α152–159 was located on the cytoplasmic surface. Therefore, M7 was proposed as a transmembrane domain between α192 and α159 (Criado et al., 1985a). M7 could form an amphipathic α helix which could line the cation channel regulated by acetylcholine binding to a site near its extracellular surface. Mishina et al. (1985) provided evidence that α141 is a site of glycosylation, presumably on the extracellular surface. Further evidence that α141 is the site of glycosylation is provided by the observation that mAbs to the synthetic peptide α127–142 are inhibited from binding to receptor by concanavalin A (Criado et al., 1986). M6 was proposed by Criado et al. (1985a) to link α141 on the extracellular surface with α152 on the cytoplasmic surface. This transmembrane domain is too short to form an α helix. It has been proposed that cysteines at α128 and α142 may be disulfide bonded to one another (Noda et al., 1982) or in a double disulfide bond with cysteines at α192 and α193 (Kao et al., 1984; Boulter et al., 1985). Evidence that mAbs to the synthetic peptide α127–143 are inhibited from binding to receptor by disulfide reducing agents and that this effect can be reversed by oxidizing agents despite affinity alkylation of the receptor at α192, α193 argues that cysteines at α128 and α142 are bonded to one another rather than in a double disulfide bond with α192, α193 (Criado et al., 1986). The N-terminus of the receptor is extracellular (Anderson et al., 1982) as is the MIR which is located somewhere between α46 and α127 (Ratnam et al., 1986).

Further studies using antibodies, in vitro mutagenesis, and crystallography should soon further refine our understanding of the transmembrane orientation of the polypeptide chains in acetylcholine receptor subunits. This should prove valuable both of itself and as a model for studying other membrane proteins.

ACKNOWLEDGMENTS

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Registry No. BOP, 56602-33-6; [Tyr-347]α330–347, 101226-00-0; [Tyr-347]α339–347, 101226-01-1; [Tyr-365]α349–365, 101248-05-9; [Tyr-365]α357–365, 101248-06-0; [Tyr-379]α360–379, 101248-07-1; [Tyr-379]α370–379, 101226-02-2; [Tyr-386]α371–386, 101226-03-3; [Tyr-386]α379–386, 101226-04-4; [Tyr-409]α389–409, 101248-08-2; [Tyr-409]α396-409, 101226-05-5; [Tyr-409]α403–409, 101226-06-6; Boc-Tyr (2,6-dichlorobenzyl), 40298-71-3; di-Boc-Hi (dicyclohexylamine salt, 31687-58-8; Boc-Asp (*O*-benzyl), 7536-58-5; Boc-Lys (2-chlorobenzylcarbonyl), 54613-99-9; Bo-Glu (*O*-benzyl), 13574-13-5; Boc-Asn (xanthenyl), 65420-40-8; Boc-Ser (benzyl), 23680-31-1; Boc-Trp, 13139-14-5; Boc-Met, 2488-15-5.

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