Location of Antigenic Determinants on Primary Sequences of Subunits of Nicotinic Acetylcholine Receptor by Peptide Mapping†

Manohar Ratnam,‡ Peter B. Sargent,§ Virender Sarin,∥ J. Lawrence Fox,∥ Dung Le Nguyen,‡ Jean Rivier,‡ Manuel Criado,‡ and Jon Lindstrom*,‡

The Salk Institute for Biological Studies, San Diego, California 92138, Department of Cell Biology, Stanford University School of Medicine, Stanford, California 94305, and Abbott Laboratories, Diagnostics Division, Abbott Park, North Chicago, Illinois 60064

Received October 9, 1985; Revised Manuscript Received December 10, 1985

ABSTRACT: The binding domains of 28 monoclonal antibodies (mAbs) against the α, β, and δ subunits of the *Torpedo* acetylcholine receptor were mapped on the primary sequences of these subunits. Small peptide fragments (2000–20 000 daltons) of the purified subunits were obtained by digestion with staphylococcal V8 protease and papain, separated on a discontinuous polyacrylamide gel electrophoretic system, and electroblotted onto diaminophenyl thioether paper. The blots were probed with the various monoclonal antibodies and also with antibodies against carboxy-terminal decapeptides of the α, β, and δ subunits to identify the carboxy-terminal fragments. From inspection of the binding patterns of the various antibodies to the subunit fragments and the molecular weights of these fragments, and by using the carboxy termini of the subunits as reference points, it was possible to deduce the regions on the primary sequence of each subunit in which the antibodies bound and in some cases to order the binding sites within these sequences. mAb 148, which inhibits receptor function by cross-linking receptor molecules on the cytoplasmic side, was mapped to the sequence β368–406. The main immunogenic region of the native receptor, which is of pathological importance in the autoimmune disease myasthenia gravis, was mapped by using mAb 210 to within 80 amino acid residues (α46–127). The overall antigenic structure of α subunits was examined. Synthetic peptides have been used to locate determinants responsible for 83% of the antibodies in antisera to denatured α subunits and 46% of the antibodies to denatured α subunits in antisera to intact receptor. Theoretical models of the transmembrane orientation of the subunit polypeptide chains were tested by determining whether mapped monoclonal antibodies bound to the extracellular or intracellular surface of receptor-rich membranes. Our results confirm previous reports that the carboxy termini of the subunits are exposed on the intracellular surface, as is part of the region between a putative channel-forming domain (M5) and a putative membrane-spanning region (M3). However, contrary to current theoretical models, the region between M5 and the putative membrane-spanning sequence M4 also appears to be on the intracellular surface, implying that M4 and M5 are not membrane-spanning domains. This finding is investigated in greater detail in our accompanying report [Ratnam, M., Nguyen, D. L., Rivier, J., Sargent, P., & Lindstrom, J. (1986) *Biochemistry* (following paper in this issue)] in which synthetic peptides are used to map precisely the binding sites of antibodies which recognize the intracellular surface of α subunits.

The nicotinic acetylcholine receptor occurs in the neuromuscular junction of vertebrate skeletal muscle and fish electric organs and is composed of four types of glycoprotein subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980). The receptor acts as a cation channel in the membrane that is regulated by the neurotransmitter acetylcholine (Neher & Sakmann, 1976; Steinbach & Stevens, 1976).

Recently, concerted efforts have been made to elucidate the structure of the receptor in native membranes. Electron microscopy, low-resolution X-ray diffraction, and neutron diffraction studies have provided useful information about the shape of the receptor in the membrane (Cartaud et al., 1978; Sealock, 1982; Kistler & Stroud, 1981; Zingsheim et al., 1980; Bon et al., 1984; Klymkowsky & Stroud, 1979; Wise et al., 1979; Brisson & Unwin, 1985). Electrophysiological data suggest that the cation channel is a water-filled pore, probably lined with hydrophilic amino acid residues (Huang et al., 1978; Lewis & Stevens, 1979; Lewis, 1979; Horn & Stevens, 1980). The recent elucidation of the complete amino acid sequences of all the receptor subunits (Noda et al., 1982, 1983a,b; Claudio et al., 1983; Sumikawa et al., 1982; Devillers-Thiery et al., 1983) has enabled more detailed studies in terms of the organization of the individual polypeptide chains of the native receptor.

The significant sequence homology among the four types of receptor subunits strongly suggests that all of them have a fundamentally similar structure and are arranged symmetrically around a central ion channel, all contributing to the formation of the channel. This has been suggested most recently by electron imaging techniques (Brisson & Unwin, 1985). All of the subunits have amino-terminal signal sequences (Noda et al., 1983a,b), and in the case of δ subunits,

† This work was supported by grants to J.L. from the National Institutes of Health (NS11323), the Muscular Dystrophy Association, the Alexander Onassis Public Benefit Foundation, and the Los Angeles and California Chapters of the Myasthenia Gravis Foundation and to P.B.S. from the National Science Foundation (BNS 8305694). M.R. was supported by a postdoctoral fellowship from the Muscular Dystrophy Association. M.C. was supported by an Osserman Fellowship from the Myasthenia Gravis Foundation.

* Correspondence should be addressed to this author.
‡ The Salk Institute for Biological Studies.
§ Stanford University School of Medicine.
∥ Abbott Laboratories.

0006-2960/86/0425-2621$01.50/0

the amino terminus (N-terminus)[1] after cleavage of the signal sequence has been shown to be on the extracellular surface (Anderson et al., 1982).

Initially, two types of theoretical models were proposed for the transmembrane orientation of the polypeptide chain in receptor subunits. According to one model (Noda et al., 1983a; Claudio et al., 1983; Devillers-Thiery et al., 1983), there are four α-helical transmembrane domains in each subunit, formed by the four most hydrophobic stretches in the primary sequence and named, starting from the N-terminus, M1, M2, M3, and M4. In this model, both the N- and C-termini are on the extracellular side. A second theoretical model (Guy, 1983; Finer-Moore & Stroud, 1984) includes, in addition to M1–M4, a fifth amphipathic transmembrane domain (M5) between M3 and M4. This domain was predicted to form an α helix with polar residues on one side and hydrophobic residues on the other, so that corresponding domains from the five subunits could come together like barrel staves to form a channel with a hydrophilic lining. Evidence is now available to show that the C-termini are intracellular (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985). Since there is evidence that the N-terminus is extracellular (Anderson et al., 1982), this is consistent with the five membrane crossing model, or any other model with an odd number of transmembrane domains. In fact, evidence has been found for two additional amphipathic transmembrane domains (M6 and M7) between the N-terminus and M1 (Criado et al., 1985a).

One approach to determining the transmembrane orientation of the polypeptide chain in receptor subunits is to determine on which side of the membrane monoclonal antibodies (mAbs) specific for defined sequences bind (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Criado et al., 1985a,b; La Rochelle et al., 1985; Young et al., 1985). By mapping the binding sites of mAbs which interfere with receptor function, it should also be possible to relate structure to function. Determination of the antigenic structure of the receptor is also important as an end in itself because the receptor is the auto-antigen for the antibody-mediated autoimmune response which impairs neuromuscular transmission in the disease myasthenia gravis (Lindstrom, 1985). In this respect, an antigenic region of critical interest is the main immunogenic region (MIR) on the extracellular surface of the α subunit, against which more than half the antibodies elicited by native receptor are directed (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1983).

We have generated a library of over 200 anti-receptor mAbs (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1983, 1985; Lindstrom et al., 1984; Criado et al., 1985a,b; S. Hochschwender et al., unpublished results), many of which bind to denatured subunits (Tzartos et al., 1985; Gullick & Lindstrom, 1983). The transmembrane orientation of binding for many of these mAbs is already known (Anderson et al., 1983, Sargent et al., 1984). A few of these mAbs noncompetitively inhibit receptor function (Lindstrom et al., 1981b; Wan & Lindstrom, 1985; Blatt et al., 1986). Identification of the primary sequences of the receptor subunits to which these mAbs bind would obviously permit us to locate these sequences in the structure of the membrane-bound receptor and in some cases to assign functional and pathological roles for these sequences.

In this paper, we report mapping of the antigenic determinants of a number of mAbs to the α, β, and δ subunits. Our results suggest that M4 and M5 are not transmembrane domains. In the following paper (Ratnam et al., 1986), we use synthetic peptides corresponding to parts of α subunits suggested to be antigenic by the peptide mapping experiments reported here to precisely map mAbs, and we use these mAbs to determine the transmembrane orientation of the sequences to which they bind. These experiments extend the mapping described here and confirm our conclusion that M4 and M5 are not transmembrane domains.

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 (Elliott et al., 1980). These vesicles were permeabilized to antibody molecules by alkali treatment as described by Neubig et al. (1979) except that the vesicles were treated with pH 11.2 instead of pH 11.0. Alternatively, vesicles were permeabilized by treatment with lithium diiodosalicylate (LIS) or saponin (Froehner et al., 1983).

Purification of α, β, and δ Subunits. Subunits were purified by preparative gel electrophoresis as described earlier (Lindstrom et al., 1979a).

Iodinations. Goat anti-rat immunoglobulin was labeled with ^{125}I by using chloramine T to a specific activity of (4–5) $\times$ 10^{18} cpm/mol (Lindstrom et al., 1981a).

Antisera against the C-Termini of the δ and α Subunits. Synthetic peptides corresponding to the 10 amino acid sequence preceding the penultimate residue of the δ subunit [[Tyr-489]δ489–499, sequence YPFDYSSDHPR] and the C-terminal decapeptide of the α subunit [[Tyr-427]α427–437, sequence YGRLIELSQEG] having a tyrosine residue attached to the amino terminus for coupling to carrier protein were purchased from Bachem (Los Angeles, CA). The peptides were coupled to keyhold limpet hemocyanin by using bis-diazotized benzidine and injected into rats, all as described earlier (Ratnam & Lindstrom, 1984). Antisera from the immunized rats with the highest antipeptide titers (Ratnam & Lindstrom, 1984) were used in further experiments.

mAbs against the N- and C-Termini of the α Subunit. Rats were immunized with the synthetic peptides [Tyr-11]α1–11 (sequence SEHETRLVANY) and [Tyr-427]α427–437 (sequence YGRLIELSQEG) as described before (Ratnam & Lindstrom, 1984). Hybridoma cell lines were obtained by fusing the mouse myeloma cell line S194 with spleen cells from an immunized rat as described (Tzartos & Lindstrom, 1980; Hochschwender et al., 1985). mAb 245 was produced against [Tyr-11]α1–11, and mAb 252 was produced against [Tyr-427]α427–437.

Antisera and mAbs against the Receptor and Its Subunits. All antisera and mAbs used were prepared in rats. The preparation of mAbs 6, 8, 10, 13, and 19 to receptors from *Torpedo* electric organ is described in Tzartos and Lindstrom (1980). The preparation of mAb 61 to receptors from *Electrophorus* electric organ is described in Tzartos et al. (1981). mAbs 237 and 254 to the synthetic peptides *Torpedo* α152–167 and α235–242 are described in Criado et al. (1985a,b). The preparation of the remaining mAbs to receptor from *Torpedo* electric organ (111, 125, 128, 129, 130, 131, 137, 141, 147, 148, 152, 153, 155, 163, 164, 169, 170, and 172) is

[1] Abbreviations: DPT, diaminophenyl thioether; ELISA, enzyme-linked immunosorbant assay; Ig, immunoglobulin; LIS, lithium diiodosalicylate; mAb, monoclonal antibody; MIR, main immunogenic region; SDS, sodium dodecyl sulfate; C-terminus, carboxy terminus; N-terminus, amino terminus; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Da, dalton(s); TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

described in Tzartos et al. (1985a). The anti-MIR mAbs 208 and 210 were derived from rats immunized with a mixture of receptors from fetal bovine muscle and the mouse muscle cell line BC3H-1 (S. Hochschwender et al., unpublished results). mAb 258 to the synthetic peptide α127–143 was prepared as described (Criado et al., 1986). Antisera to the subunits were described in Lindstrom et al. (1979a,b).

Peptide Mapping of α, β, and δ Subunits. Small peptide fragments of the subunits (2000–20 000 daltons) were obtained by incubating the subunit (10–20 μg) with staphylococcal V8 protease (Miles) (2.5 μg) in 50 μL of 0.1 M sodium phosphate buffer (pH 7.5)/0.3% SDS/10% glycerol or with papain (Sigma) (5 μg) in 0.1 M Tris-HCl buffer (pH 6.8)/0.3% SDS/10% glycerol overnight at 37 °C. The proteolysates were then mixed with 2% SDS and 5% β-mercaptoethanol and boiled. The samples were then run in a discontinuous electrophoretic system in 12.5% acrylamide gels, as reported by Kyte and Rodriguez (1983), with some modifications [specifically, an acrylamide: *N,N'*-methylenebis(acrylamide) ratio of 10:1 in the stacking and separating gels, 15 μL of TEMED in the separating gel and 40 μL in the stacking gel, and 100 μL of ammonium persulfate in the stacking gel]. This electrophoretic system efficiently resolved peptides in the range 1700–18 000 Da as sharp bands. Plots of log molecular weight vs. mobility were linear for marker peptides of 1695–17 201 Da (myoglobin fragments from Pharmacia).

Digestion of the α subunit with trypsin was carried out by incubating 30 μg of the purified subunit with 0.2 μg of trypsin in 0.1 M sodium phosphate buffer, pH 7.5, and 0.5% Triton X-100 for 90 min at 22 °C. The reaction was stopped by adding 2.0% SDS and 0.1 M β-mercaptoethanol and boiling for 5 min. The sample was electrophoresed on an SDS/15.0% polyacrylamide gel.

Immunoblotting on Diaminophenyl Thioether (DPT) Paper. Aminophenyl thioether (APT) paper was prepared and activated to DPT according to Seed (1982). DPT paper was used because small peptides bind to it covalently, whereas they do not bind well to nitrocellulose or to nylon membranes. DPT paper also has the advantage of being amenable to repeated probing with antibodies. Peptide maps of the α, β, and δ subunits were electroblotted onto the paper at 4 °C and probed with antibodies, essentially as described earlier (Gullick & Lindstrom, 1983). The blots were cut into strips which were incubated with shaking in 50-mL tubes with antisera (1:500–1:1000 dilution) or with various mAbs (1–10 nM) in 5–10 mL of 10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl/0.25% gelatin/0.5% Triton X-100 overnight at 22 °C followed by washing in the buffer and incubation with affinity-purified ^{125}I-labeled goat anti-rat immunoglobulin (0.1–0.5 nM) for an additional 2 h. The blots were finally washed and autoradiographed. For repeated probing of the DPT paper strips with antisera against whole subunits, the paper was incubated with 2 × 200 mL of 0.05 M sodium phosphate buffer (pH 7.5)/0.1 M β-mercaptoethanol/2% SDS at 60 °C for 4 h. This resulted in almost complete removal of the initial mAb probe.

ELISA. The binding of mAb 125 to receptor immobilized in microtiter dishes (Immunolon I, Dynatech, Alexandria, VA) was assayed by using glucose oxidase labeled anti-antibody as described earlier (Hochschwender et al., 1985). Inhibition of this binding by various concentrations of detergent-solubilized receptor or receptor in membranes was monitored by measuring the absorbance at 410 nm.

Assay of Anti-Peptide Titers in Antisera to the α Subunit and to Whole Receptor. Synthetic peptides of the α subunit (Lindstrom et al., 1984; Criado et al., 1985a,b, 1986; Ratnam et al., 1985) were labeled with ^{125}I (Lindstrom et al., 1981a). Antisera against whole receptor as well as against the α subunit were assayed by incubating each ^{125}I-labeled peptide (5 nM) with the antisera (0.5–5 μL) in 100 μL of 10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl/0.5% Triton X-100 and precipitating the antigen–antibody complex with goat anti-rat immunoblogulin, all as described earlier (Lindstrom et al., 1981a).

Electron Microscopy. The transmembrane orientation of epitopes recognized by specific mAbs was determined by using permeabilized, receptor-rich vesicles immobilized to the bottoms of poly(vinyl chloride) assay wells as described by Wray and Sealock (1984). This technique was learned during a visit by P.B.S. to Dr. Robert Sealock's laboratory at The University of North Carolina. Membranes, which were purified from *Torpedo* electric organ by the method of Sobel et al. (1977) and rendered permeable to macromolecules by treatment with high pH, were provided by Dr. Sealock. The orientation of mAb binding was determined by reference to the binding to cobra toxin, which recognizes the extracellular domain of the receptor. A typical experiment was carried out as described by La Rochelle et al. (1985) and entailed incubating membranes in sequence with a mAb, with rabbit anti-rat Ig, with 6-nm colloidal gold–protein A, with cobra toxin, with rabbit anti-cobra toxin Ig, and with 15-nm colloidal gold–protein A.

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

To map the binding specificities of a number of antisubunit mAbs, the following strategy was adopted. First, small fragments (2000–20 000 Da) of the purified subunits were obtained by extensive digestion with staphylococcal V8 protease or papain. The digests were run on high-resolution SDS–polyacrylamide gels and electroblotted onto DPT paper. The blots were cut into strips and probed with the various mAbs and also with antibodies specific to the C-terminal decapeptide of each subunit, i.e., antisera against the C-termini of the δ and α subunits (Ratnam & Lindstrom, 1984), or mAb 252 to the C-terminus of α subunits, or mAbs 170 and 172 which cross-reacted with the C-terminal decapeptide of the β subunit (Lindstrom et al., 1984). The apparent molecular weights of fragments that bound the antibodies to the C-terminus were determined, and from these values, the lengths of the polypeptide chains were calculated by assuming a mean residue weight of 115. The two limit positions within which an mAb binding site could be mapped were determined by the number of amino acid residues (S) in the smallest C-terminal fragment that bound this mAb, the number of residues (L) in the largest C-terminal fragment that did not bind this mAb, and the total number of residues (T) in the subunit. The positions of the two limit residues would be $T - S$ and $T - L$. Since it was possible that the marker antibodies might bind to only the penultimate six residues of the C-terminal decapeptide, four residues were further subtracted from $T - S$. In all the blotting experiments, the peptide bands were identified by probing with antisera against the whole subunit subsequent to probing with an mAb. Blots of protease controls (without subunit) did not show any bands. The specificity of binding of mAbs to blots was confirmed by the inability of antibodies in normal rat sera to bind to the peptide bands. The γ subunit was not mapped in this study because peptide maps of this

Table I: mAb Binding Properties of Carboxy-Terminal Fragments of the δ Subunit

protease	fragment mol wt (×10⁻³)	anti-δ C-terminal serum	mAbs 131	129	130	128	137	141	166
papain	28.4	+	+	+	+	+	+	+	+
papain	25.0	+	+	+	+	+	+	+	+
papain	21.0	+	+	+	+	+	+	+	+
papain	10.4	+	+	+	+	+	–	–	–
V8	9.4	?	+	+	+	+	–	–	–
V8	6.5	+	+	+	+	+	–	–	–
V8	5.4	+	+	+	+	+	–	–	–
V8	4.3	+	+	+	+	+	–	–	–

Table II: mAb Binding Properties of Carboxy-Terminal Fragments of the β Subunit

protease	fragment mol wt (×10⁻³)	mAbs 170	172	125	111	148	10	169
V8	30.6	+	+	+	+	+	+	+
V8	14.8	+	+	+	+	+	+	+
papain	11.2	+	+	+	+	+	+	–
V8	7.3	+	+	+	–	–	–	–
V8	4.1	+	+	+	–	–	–	–
V8	3.2	+	+	–	–	–	–	–
V8	2.7	+	+	–	–	–	–	–

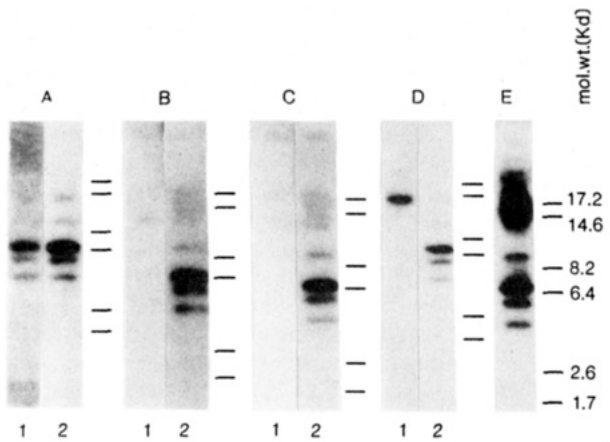

FIGURE 1: Immunoblots of peptide maps from V8 protease digests of the δ subunit. Peptides obtained by digesting 10 μg of the δ subunit with 2.5 μg of V8 protease were electrophoresed and blotted on DPT paper. The paper was cut into longitudinal strips and probed with various mAbs. Strips from the same gel are grouped together (groups A–E), and the positions of molecular weight standards corresponding to each group are indicated by dark lines on the right. The primary antibody probes used are antiserum to the C-terminus of δ subunits (A1), mAb 128 (A2), mAb 141 (B1), mAb 129 (B2), mAb 166 (C1), mAb 130 (C2), mAb 137 (D1), mAb 131 (D2), and antiserum to the δ subunit (E).

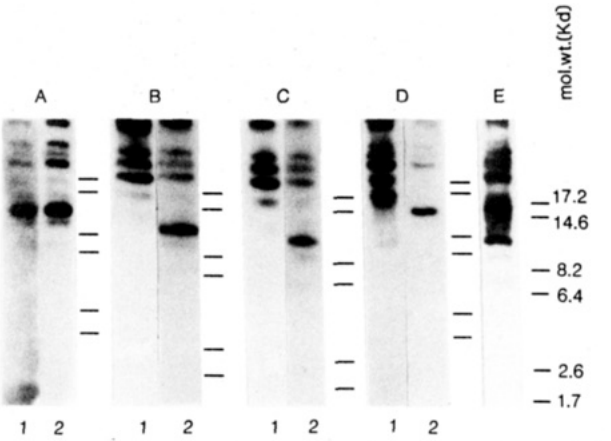

FIGURE 2: Immunoblots of peptide maps from papain digests of the δ subunit. Peptides obtained by digesting 10 μg of the δ subunit with 5 μg of papain were electrophoresed and blotted on DPT paper. The primary antibody probes used are antiserum to the C-terminus of δ (A1), mAb 128 (A2), mAb 141 (B1), mAb 129 (B2), mAb 166 (C1), mAb 130 (C2), mAb 137 (D1), mAb 131 (D2), and antiserum to the δ subunit (E).

subunit often gave diffuse bands, perhaps because of the presence of multiple proteolytic cleavage sites very close to each other.

Peptide Maps of the δ Subunit. Figure 1 shows electroblots of peptide maps of the δ subunit obtained with staphylococcal V8 protease. Among the fragments that were revealed by using antiserum to δ subunits, the three smallest fragments (6.5, 5.4, and 4.3 kDa) clearly bound antibodies against the C-terminal decapeptide of the δ subunit. Among the anti-δ-subunit mAbs tested, mAbs 128, 129, 130, and 131 bound to all three fragments, indicating that these mAbs bind within 4.3 kDa from the C-terminus (Figure 1, Table I). mAbs 141, 166, and 137, on the other hand, did not bind to any of the fragments (Figure 1, Table I).

Immunoblots of peptide fragments of the δ subunits obtained by digestion with papain are shown in Figure 2. It can be seen that a number of bands (28.4, 25.0, 21.0, and 10.4 kDa) bound antibodies to the C-terminus, the 10.4-kDa band being the most prominent. mAbs 128, 129, 130, and 131 bound to the 10.4-kDa band (Figure 2, Table I), and this result is consistent with the previous results with V8 protease digests. mAbs 141, 166, and 137 bound only to the C-terminal fragments of 21.0–28.4 kDa but not to the 10.4-kDa fragment (Figure 2, Table I), indicating that these mAbs bind between 10.4 and 21.0 kDa from the C-terminus of the δ subunit.

Among mAbs 141, 166, and 137, identical peptide binding patterns were obtained with mAbs 141 and 166 (Figures 1 and 2). mAb 137, on the other hand, bound to a 14.0-kDa fragment (V8 protease) and also to nonterminal 14.5- and 17.5-kDa fragments (papain) of the δ subunit to which the other mAbs did not bind, indicating that this mAb binds to a different site than do mAbs 141 and 166.

Peptide Maps of the β Subunit. Peptide maps of the β subunit obtained by digestion with V8 protease followed by immunoblotting are shown in Figure 3. A number of these fragments (2.7, 3.2, 4.1, 7.3, 14.8, and 30.6 kDa) bound mAbs 170 and 172 which were previously shown to cross-react with the C-terminal decapeptide of the β subunit (Lindstrom et al., 1984) (Figure 3, Table II). The smallest set of these fragments (2.7–4.1 kDa) closely resembled the pattern of C-terminal fragments produced in peptide maps of the δ subunit

Table III: mAb Binding Properties of Papain-Generated Carboxy-Terminal Fragments of the α Subunit

fragment mol wt ($\times 10^{-3}$)	anti-α C-terminal serum	mAbs										
		19	147	8	61	153	152	155	164	149	13	10
22.7	+	+	+	+	+	+	+	+	+	+	+	+
19.0	+	+	+	+	+	+	+	+	+	+	+	+
14.8	+	+	+	+	+	+	+	+	+	+	+	+
11.5	+	+	+	+	+	+	+	+	+	+	+	+
5.9	+	–	–	–	–	–	–	–	–	–	–	–

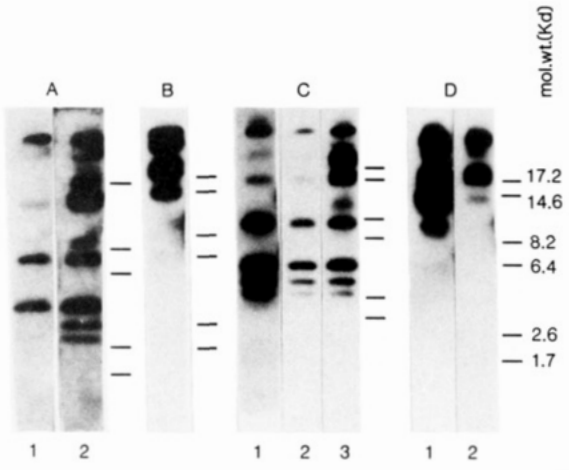

FIGURE 3: Immunoblots of peptide maps from V8 protease digests of the β subunit. Peptides obtained by digesting 10 μg of the β subunit with 2.5 μg of V8 protease were electrophoresed and blotted on DPT paper. The primary antibody probes used are mAb 125 (A1), antiserum to β subunits (A2), mAb 111 (B), mAb 170 (C1), mAb 172 (C2), antiserum to β subunits (C3), mAb 169 (D1), and mAb 148 (D2). The binding pattern of mAb 10 was identical with that of mAb 148 (D2).

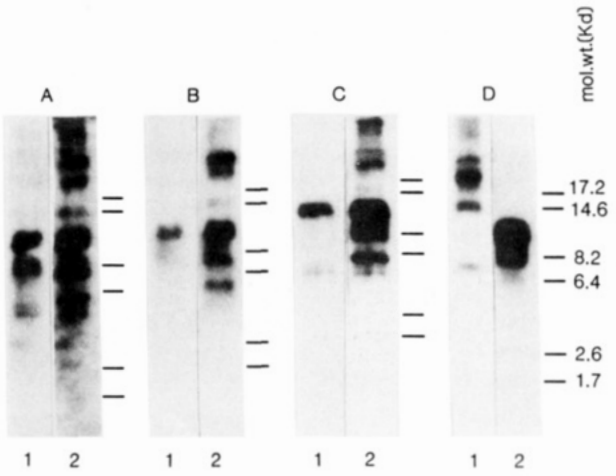

FIGURE 4: Immunoblots of peptide maps from papain digests of the β subunit. Peptides obtained by digesting 10 μg of the β subunit with 5 μg of papain were electrophoresed and blotted on DPT paper. The primary antibody probes used are mAb 125 (A1), antiserum to β subunits (A2), mAb 111 (B1), antiserum to β subunits (B2), mAb 170 (C1), antiserum to β subunits (C2), mAb 169 (D1), and mAb 148 (D2). The binding pattern of mAb 10 was identical with that of mAb 148 (D2).

(Figure 1, Table I). The actual molecular weights of the δ-subunit fragments are, however, greater by about 2000 Da than the corresponding β-subunit fragments. This is easily explained by the fact that, on alignment of homologous amino acid sequences of the β and δ subunits, the carboxy terminus of the δ subunit extends about 1500 Da beyond that of the β subunit (Noda et al., 1983). mAb 125 bound to all the C-terminal fragments, except the 2.7- and 3.2-dDa fragments (Figure 3, Table II). This antibody should, therefore, bind between 3.2 and 4.1 kDa from the C-terminus of the β subunit. mAbs 111, 148, 10, and 169 bound only to the 30.6- and 14.8-kDa C-terminal fragments (Figure 3, Table II), indicating that these antibodies cannot bind within 7.3 kDa from the C-terminus.

Immunoblots of peptide maps of the β subunit obtained by digestion with papain (Figure 4) show a single prominent band (11.2 kDa) that can bind the anti-C-terminal mAbs 170 and 172. mAbs 125, 111, 148, and 10 bound to this fragment (Figure 4, Table II). From this and the preceding data with V8 protease digests of the β subunit, we can conclude that mAbs 111, 148, and 10 should bind between 7.3 and 11.2 kDa from the C-terminus. Further, mAb 169 did not bind to the 11.2-kDa C-terminal fragment (Figure 4, Table II), so that the binding site of this mAb should be between 11.2 and 14.5 kDa from the C-terminus.

The presence of relatively large, non-C-terminal fragments of the β subunit (25.7, 21.5, and 15.6 kDa) that bind mAb 169 but not mAbs 111, 148, and 10 (Figures 3 and 4) shows that mAb 169 should bind to the N-terminal side of mAbs 111, 148, and 10 on the β-subunit sequence, since mAbs 111 and 148 bound within 14.8 kDa from the C-terminus. Similarly, the binding of mAb 111 to 25.1- and 14.5-kDa fragments to which mAbs 148 and 10 did not bind (Figures 3 and 4) indicates that mAb 148 should bind on the C-terminal side of mAb 111.

Peptide Maps of the α Subunit. The peptide map of the α subunit generated with staphylococcal V8 protease closely resembled that produced with the same enzyme from the δ subunit (Figure 1, Table I) in that it showed small fragments of molecular weights 2.3K, 3.1K, 4.1K, and 5.7K (figure not shown). However, it was not possible to obtain any useful data on antigenic mapping using these fragments because the smallest fragments were too faint to produce an unambiguous signal and also because of the presence of multiple comigrating bands above 6 kDa. Proteolysis of the α subunit with papain gave peptides of >30 kDa and also a number of small fragments that bound antibodies against the C-terminal decapeptide of the α subunit on blots. The C-terminal peptides had molecular weights of 22.7K, 19.0K, 14.8K, 11.5K, and 5.9K (Figure 5, Table III). The anti-α-subunit mAbs, 19, 147, 8, 61, 152, 155, 164, 153, 149, 13, and 10, bound to all the C-terminal fragments except the smallest (5.9 kDa) fragment (Figure 6, Table III), showing that these mAbs bind between 5.9 and 11.5 kDa from the C-terminus. The binding sites of these mAbs are mapped more precisely in the following paper (Ratnam et al., 1986).

Mapping of the MIR on the α Subunit. The MIR is a conformationally dependent antigenic determinant, and antibodies to it seldom react with the denatured α subunit with high affinity. Antibodies to the MIR are defined by mutually competitive binding to receptors. Among the mAbs against the MIR tested by us, mAbs 210, 208, and 6 bound specifically to denatured α subunits on blots, but only when used at a relatively high concentration (10–100 nM). The specificity of the binding of mAbs 210, 208, and 6 to α subunits on Western blots containing all four receptor subunits is illustrated

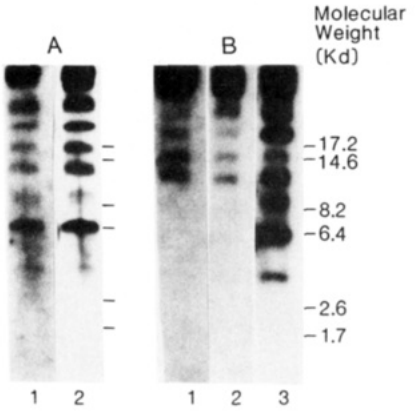

FIGURE 5: Immunoblots of peptide maps from papain digests of the α subunit. Peptides obtained by digesting 10 μg of the α subunit with 5 μg of papain were electrophoresed and blotted on DPT paper. The primary antibodies used were antiserum to the C-terminus of α subunits (A1), antiserum to α subunits (A2), mAb 13 (B1), mAb 155 (B2), and antiserum to α subunits (B3). The binding patterns of other anti-α-subunit mAbs mapped, i.e., mAbs 19, 147, 8, 61, 153, 152, 164, 149, and 10, were similar to those shown in this figure for mAbs 13 and 155.

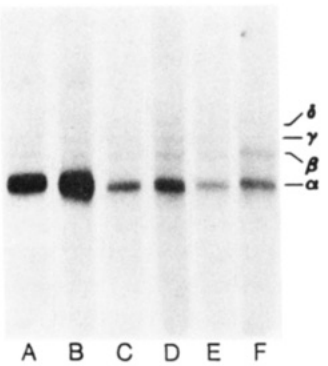

FIGURE 6: Binding of anti-MIR mAbs 210, 208, and 6 on Western blots of the receptor. Receptor (~5 μg per lane) were electrophoresed on an SDS–10.0% polyacrylamide gel and blotted on DPT paper. Strips from the blot were probed with the mAbs followed by ^{125}I-labeled goat anti-rat IgG. The primary mAbs used are mAb 210, 10 nM (A); mAb 210, 100 nM (B); mAb 208, 10 nM (C); mAb 208, 100 nM (D); mAb 6, 10 nM (E); and mAb 6, 100 nM (F). the positions of the α-, β-, γ-, and δ-subunit bands are indicated in the figure.

in Figure 6. Since mAb 210 gave the strongest signal on immunoblots, this mAb was chosen to map the MIR sequence on the α subunit.

It was previously reported that the MIR sequence is present within a 19-kDa peptide of the α subunit generated with staphylococcal V8 protease (Gullick et al., 1981) which begins at residue α46 (Ratnam et al., 1986b). Since most of the fragments obtained in our low molecular weight peptide maps were small C-terminal fragments, large fragments which included the N-terminal half of the α subunit were generated in order to map the binding site of mAb 210. Figure 7 shows Western blots of peptide fragments of the α subunit obtained by digestion with trypsin and resolved on Laemmli gels. Under our conditions of proteolysis, two major bands were obtained with molecular weights of 25K and 33K. Neither of these fragments contained the N- or C-terminus of the α subunit, since mAbs 245 and 252, specific for the sequences α1–10 and α428–437, respectively, did not bind to them. Both fragments bound mAbs 258, 237, and 254, which are specific for the peptides α127–143 (Criado et al., 1986), α152–159 (Criado et al., 1985a), and α235–242 (Criado et al., 1985b), respectively, indicating that they contain the sequence α127–242.

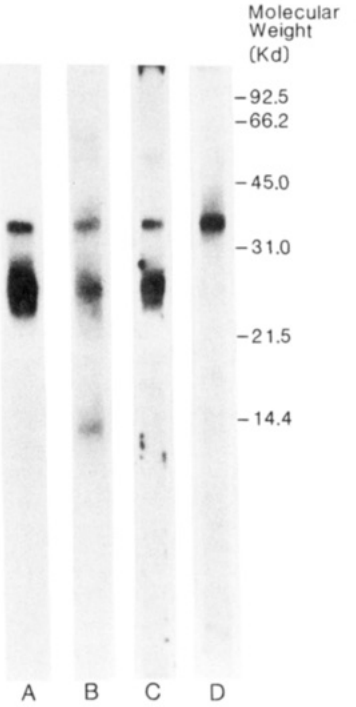

FIGURE 7: Immunoblots of tryptic maps of the α subunit. Peptide fragments obtained by digesting the purified α subunit (30 μg) with trypsin (0.2 μg) were electrophoresed in a wide lane on an SDS–15.0% polyacrylamide gel and blotted on DPT paper. Strips from the blot were probed with mAb 258 (A), mAb 237 (B), mAb 254 (C), and mAb 210 (D). The molecular weight standards (Bio-Rad) used are indicated on the right.

mAb 210 bound to the 33-kDa fragment but not to the 25-kDa fragment, indicating that its binding site should not include the sequence α127–242. Since the 19-kDa fragment generated by V8 protease and starting at residue α46 (Ratnam et al., 1986b) did not bind mAb 254 but did bind mAb 210 (data not shown), it follows that mAb 210 should bind on the N-terminal side of the residue α128; i.e., the MIR is located between α46 and α127.

Summary of Results of Mapping mAb Binding Sites. The results of the mapping experiments described in the preceding sections are summarized in Figure 8. The regions within which the binding sites of the mAbs can be mapped are indicated below representations of the sequences of the subunits, as are the regions corresponding to the putative transmembrane domains M1–M7. On α subunits, the region within which the MIR can be mapped is indicated as are the site of glycosylation (at α141; Mishina et al., 1985) and the site at which an acetylcholine binding site affinity labeling reagent binds (α192; Kao et al., 1984). Note that most mAbs to denatured subunits bind to sequences in relatively homologous positions near the C-terminal ends of the subunits.

Mapping the Overall Antigenic Structure of the α Subunit. We have synthetic peptides covering 35% of the primary sequence of the α subunit (Lindstrom et al., 1984; Criado et al., 1985a,b, 1986; Ratnam et al., 1986). We determined the percent cross-reactivities of antibodies in antisera to the α subunit and to whole receptor with the ^{125}I-labeled peptides by indirect immune precipitation. In Figure 9, these values are plotted as percent of the total antibody titer against denatured ^{125}I-labeled α subunit. Note that most antibodies which cross-react with synthetic peptides do so with peptides from relatively near the C-terminus. Immunogenic sequences are highly localized: 73% of the total antibody titer in antiserum to the α subunit is directed against less than 20% of the primary subunit sequence, whereas no antibodies bind to

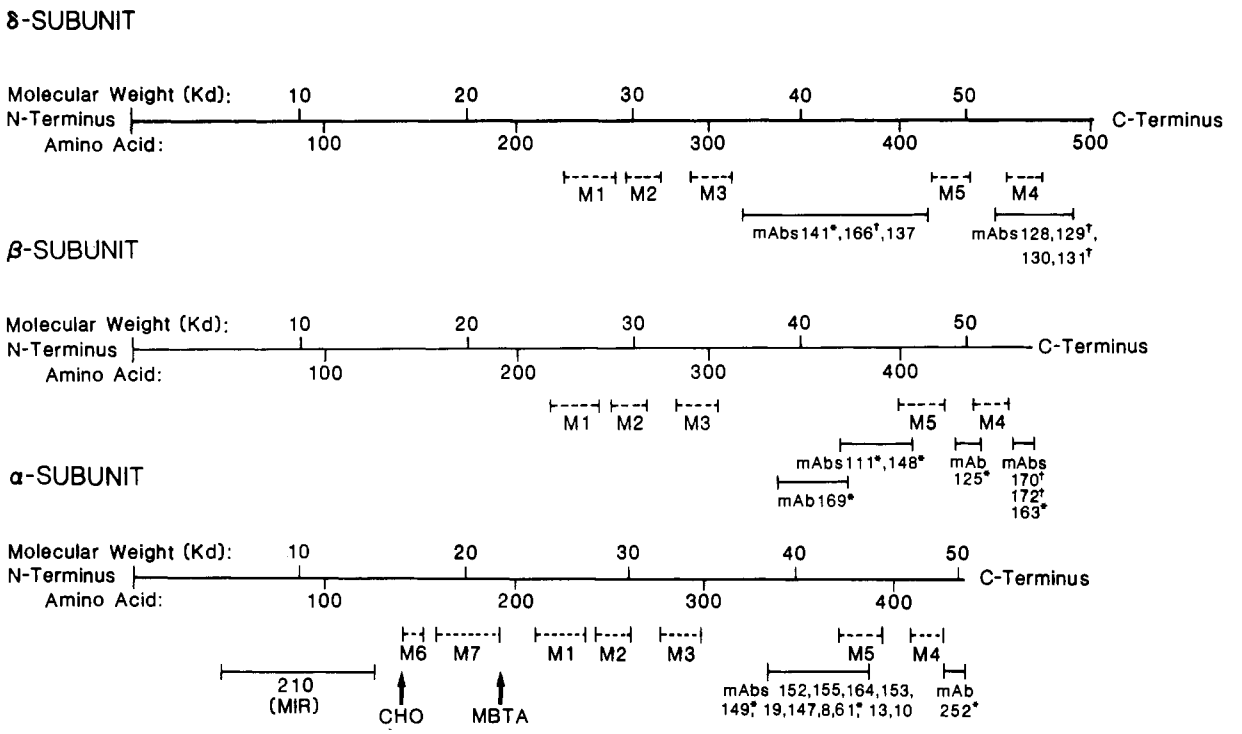

FIGURE 8: Schematic representation of mAb binding domains on δ, β, and α subunits. The subunit polypeptide chains are represented by horizontal lines, and the positions of amino acid residues as well as the molecular weights of segments starting from the amino termini are indicated. The positions of putative membrane-spanning domains (Claudio et al., 1983; Devilliers-Thiery et al., 1983; Noda et al., 1983a; Guy, 1983; Finer-Moore & Stroud, 1984) are indicated by broken lines. The presence of transmembrane domains M6 and M7 has so far been established in the α subunit alone (Criado et al., 1985a), and therefore, these domains are indicated only for this subunit. The glycosylation (CHO) (Mishina et al., 1985) and affinity alkylation (MBTA) sites (Kao et al., 1984) on the α subunit are indicated by arrows. The binding domains of various mAbs are represented by solid lines. An asterisk indicates mAbs whose binding sites have been localized on the cytoplasmic side by electron microscopy (Sargent et al., 1984; P. B. Sargent et al., unpublished results), and a dagger indicates mAbs whose binding sites were located as intracellular by other means (Anderson et al., 1982, 1983; Lindstrom et al., 1984; J. Lindstrom et al., unpublished results).

peptides corresponding to 15% of the sequence, and much of the rest of the sequence must be immunologically silent. It has been our experience that sequences which are poor antigens for antisera to α or intact receptors are also poor immunogens, and vice versa. In the anti-receptor serum studied, about a third of its titer against intact receptor [22 μmol of toxin binding sites of receptor (mostly in dimers) bound per liter, implying $\sim$5.5 μM IgG] can be accounted for by reaction with denatured subunits (3.8 μmol of subunits bound per liter, implying $\sim$1.9 μM IgG). The relative amounts of cross-reaction with the denatured α, β, γ, and δ subunits were 14, 26, 18, and 42%, respectively. In Figure 9, 46% of the anti-α-subunit antibodies in this antiserum bind to peptides corresponding to 20% of the primary subunit sequence. Presumably, much less of the immunogenicity of α subunits in intact receptor (46%) is accounted for by the peptides which have been synthesized than in the case of antisera to denatured α subunits (83%), because in antisera to native receptor at least half of the antibodies are directed at the MIR.

Transmembrane Orientation of the Polypeptide Chain. Mapping the binding sites of the mAbs produced a very interesting result. The binding site for mAb 125 was localized to somewhere between 3.2×10^3 and 4.1×10^3 daltons from the C-terminus of β, which corresponds to the sequence β429–441. This sequence was predicted by Guy (1983) and by Finer-Moore and Stroud (1984) to be part of a short extracellular domain between the putative amphipathic transmembrane M5 and the putative hydrophobic domain M4. However, Anderson et al. (1983) had concluded that mAb 125 bound to an intracellular domain. Clearly, if the C-terminus as well as the sequence to which mAb 125 binds is intracellular, then both the four and the five transmembrane domain models

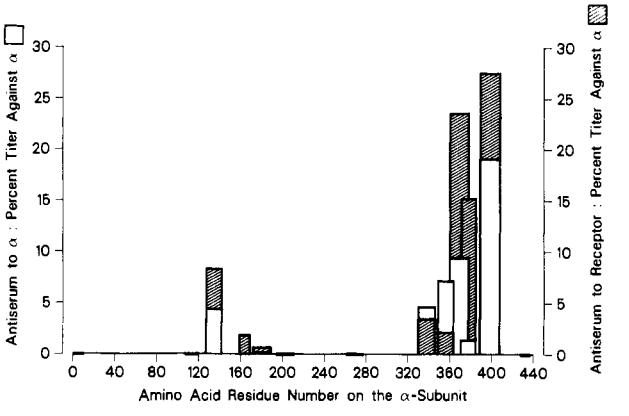

FIGURE 9: Cross-reactivities of antibodies to α subunit present in antisera to the α subunit and to whole receptor with synthetic peptides. Synthetic peptides of the α subunit were labeled with ^{125}I, and cross-reacting antibodies in an antiserum to the α subunit (open bars) and in an antireceptor serum (hatched bars) were measured by solution radioimmunoassays. Antiserum (5 μL) were incubated overnight with 100 μL of 5 nM antigen in buffer containing 0.5% Triton X-100 in triplicate. Then, anti-antibody was added for 30 min followed by dilution to 1 mL, centrifugation for 2 min in a microfuge, and two washes of the pellets. Normal serum control values were subtracted from determinations of ^{125}I in the pellets. Titers are moles of antigen bound per liter of serum. Cross-reactivities are expressed as the percent of the total antibody titer against ^{125}I-labeled α subunit in the sera. The horizontal axis of the histogram indicates the positions of amino acid residues in the primary sequence of the α subunit. The positions and lengths of the ^{125}I-peptides are represented by the positions and widths of the vertical bars.

in question must be incorrect. Therefore, first we rechecked the transmembrane orientation of the C-terminus and then of the binding site for mAb 125.

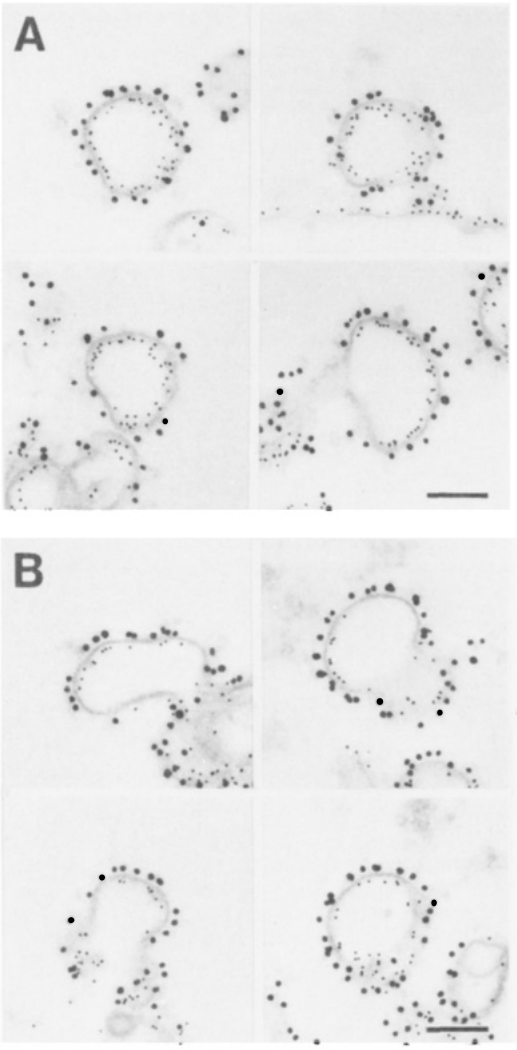

FIGURE 10: Visualization of the transmembrane orientation of the C-terminus of β and α subunits. mAb 163 (A) binds to the C-terminus of β subunits, and mAb 252 (B) binds to the C-terminus of α subunits. Both panel A and panel B show a montage of four electron micrographs of labeled receptor-rich membranes. The small gold particles indicate binding of the mAbs, and the large gold particles indicate binding of cobra toxin, which is known to bind to the extracellular domain of the receptor. Both mAb 163 and mAb 252 must recognize the intracellular domain of the receptor, since the small gold particles and the large gold particles are found on opposite sides of the membrane. Bar (lower right), 200 nm.

The transmembrane orientation of the C-terminus was determined by electron microscopy using receptor-rich vesicles as described by Wray and Sealock (1984) and La Rochelle et al. (1985). The antibodies used were mAb 163, which binds to the C-terminus of β subunits (Lindstrom et al., 1984), and mAb 252, which was made against the C-terminus of α (see Materials and Methods). mAb binding was visualized by using 6-nm colloidal gold particles and was compared to the binding of cobra toxin, which was visualized by using 15-nm colloidal gold particles and which is known to bind to the extracellular surface of the receptor. Small and large colloidal gold particles were found on opposite sides of the membranes for both mAb 163 (Figure 10A) and mAb 252 (Figure 10B), indicating that the mAbs bind to the intracellular surface of the receptor.

The transmembrane orientation of the binding site for mAb 125 was first studied immunochemically. Receptor was immobilized on microtiter plates, and the binding of mAb 125 (1 nM) was measured in the presence of increasing concentrations of detergent-solubilized receptor, native receptor-rich vesicles (with the receptor oriented right side out), or vesicles

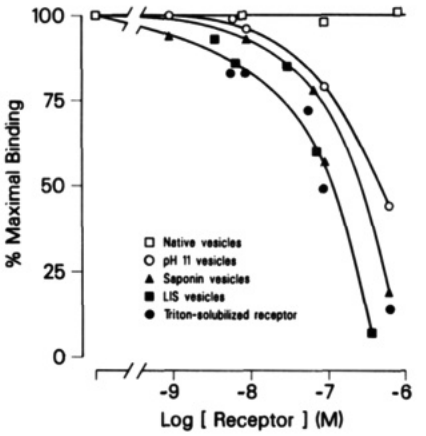

FIGURE 11: Determination of the transmembrane orientation of the binding site for mAb 125 by an immunochemical method. The binding of mAb 125 (1 nM) to receptor immobilized in ELISA wells was monitored by using glucose oxidase labeled anti-antibody, assaying for glucose oxidase, and measuring the absorbance at 410 nm in ELISA assays (see Materials and Methods). The inhibition of this binding was followed by measuring changes in the absorbance in triplicate assays in the presence of various concentrations of native receptor vesicles sealed right side out (□), vesicles permeabilized by extraction at pH 11.2 (○), vesicles permeabilized with saponin (▲), vesicles permeabilized with LIS (■), and detergent (0.5% Triton X-100) solubilized receptor (●). The results are plotted as the percent of control, i.e., in the absence of inhibiting receptor.

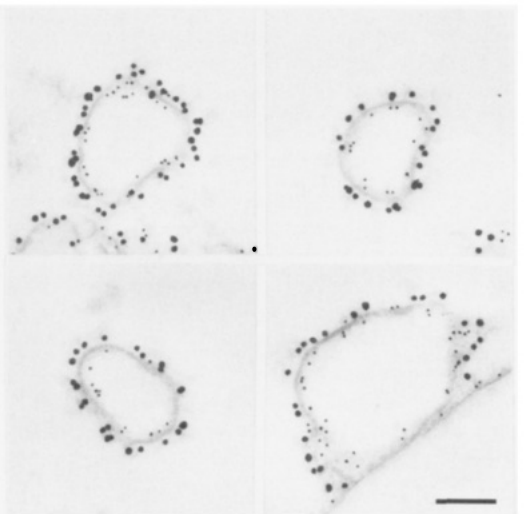

FIGURE 12: Visualization of the transmembrane orientation of the binding site for mAb 125. A montage of four electron micrographs of labeled receptor-rich membranes is shown. The small gold particles indicate binding of mAb 125, and the large gold particles indicate binding of cobra toxin, which is known to bind to the extracellular domain of the receptor. mAb 125 must recognize the intracellular domain of the receptor, since the small gold particles and the large gold particles are found on opposite sides of the membrane. Bar (lower right), 200 nm.

permeabilized by treatment with pH 11.2, LIS, or saponin. Figure 11 shows that mAb 125 did not bind to receptors in native vesicles but did bind if the intracellular surface were exposed by permeabilization of the vesicles or solubilization of the receptor.

The transmembrane orientation of the binding site of mAb 125 was also determined by the electron microscopic technique of Wray and Sealock (1984) and La Rochelle et al. (1985). Figure 12 shows that mAb 125 binds to the intracellular surface.

These experiments confirm our previous conclusions that the C-termini are on the cytoplasmic surface (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984) and that the binding

site on the β subunit for mAb 125 is on the cytoplasmic surface (Anderson et al., 1983).

Discussion

The use of peptide maps of purified subunits of the acetylcholine receptor and sequence-specific mAbs is a useful approach for mapping the binding domains of various mAbs against the receptor on its primary sequence. The usefulness of mapping the binding domains of these mAbs is 3-fold: (1) It is possible to obtain information on the transmembrane orientation of the polypeptide chains of the receptor from a knowledge of the location of the antigenic determinants on the native receptor in membranes; (2) regions on the primary sequence that have a direct role in the physiological functioning of the receptor can be identified by mapping mAbs that affect channel activity; and (3) mapping of the receptor and also the overall antigenic structure of the receptor will enable the pinpointing of peptide sequences that play a critical pathogenic role in myasthenia gravis. The sequences specificities of the various mAbs to α, β, and δ subunits mapped in this way are represented schematically in Figure 8.

Mapping mAb Binding Sites on the δ Subunit. mAbs 128, 129, 130, and 131 bind within the C-terminal 41 residues of the δ subunit (Figures 1, 2, and 8 and Table I). Since the C-terminal decapeptide has been found not to bind to these antibodies (Lindstrom et al., 1984), they should bind between residues 460 and 491 in the δ-subunit sequence. mAbs 129 and 131 have been previously reported to bind to the intracellular side of the receptor (Anderson et al., 1983). Therefore, these results provide additional evidence in support of the idea (Figure 10; Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985) that the C-termini of the receptor subunits are on the intracellular surface. mAbs 141, 166, and 137 bind between residues 318 and 415 on the δ subunit (Figures 1, 2, and 10 and Table I). This stretch is situated between M3 and M5 in previously described theoretical models of receptor structure (Finer-Moore & Stroud, 1984; Guy, 1984). mAb 141 has been shown by electron microscopy to bind to the cytoplasmic surface of the receptor in amphibian skeletal muscle, and mAb 166 similarly binds only to muscle which has been permeabilized by saponin (Sargent et al., 1984). Our results thus imply that at least part of the sequence 318–415 on the δ subunit is intracellular. This finding is consistent with previous reports that a part of the corresponding sequence is intracellular in the γ (γ360–377; La Rochelle et al., 1985) and β (β338–346; Young et al., 1985) subunits and, thus, supports the concept of homology in the transmembrane orientation of the different subunit polypeptide chains.

Mapping mAb Binding Sites on β Subunits. mAb 111 binds within the sequence 368–406 on the β subunit (Figures 3, 4, and 8 and Table II). This region extends from about the midpoint between M3 and M5 up to the beginning of M5. mAb 111 has been demonstrated by electron microscopy to bind to the intracellular side of the receptor in muscle (Sargent et al., 1984). This is consistent with our previous conclusions about the intracellular location of a corresponding sequence in the δ subunit and with other reports for the γ (La Rochelle et al., 1985) and β subunits (Young et al., 1985). The binding site of mAb 169 can be mapped to the sequence 336–372 on the β subunit (Figures 3, 4, and 8 and Table II). This mAb has been demonstrated to bind to the intracellular side of the receptor in pH 11 treated membrane vesicles from *Torpedo* electric organs using the methods of Wray and Sealock (1984) and La Rochelle et al. (1985) (Robert Sealock, personal communication). This finding also corroborates the preceding results. Anderson et al. (1983) previously studied δ subunits synthesized in dog pancreas endoplasmic reticulum vesicles and observed that mAb 169 bound to a 37-kDa glycosylated fragment protected on the interior of the vesicle from trypsin added to the exterior of the vesicle, which suggested that mAb 169 bound to the extracellular surface of the receptor. However, since subunits synthesized in this way do not achieve their native conformation (e.g., the α subunit does not bind α-bungarotoxin with high affinity), it may be that the domain recognized by mAb 169 crosses the membrane in a later conformational maturation step. More likely, the domain recognized by mAb 169 may always reside on the intracellular surface but, unlike the case of mAb 125, there may be no tryptic cleavage site between this domain and the extracellular domain. All models of the transmembrane orientation of subunit polypeptide chains predict at least two intracellular domains within 37 kDa of the N-terminus.

Determining the Transmembrane Orientation of Mapped Sequences. The sequence specificity of mAb 125 on the β subunit is particularly interesting. The binding site for mAb 125 can be mapped to a narrow region, between residues 429 and 441 on the β subunit (Figures 3, 4, and 8 and Table II). Localization of the binding site in this region should be quite accurate because the information for this is derived from very small C-terminal fragments, among which a difference of a few amino acid residues would lead to detectable differences in mobility in the electrophoretic system used in our study. Even if there were a wide margin of error (β400–450), our basic conclusion would not be altered. This region lies between the putative channel-forming domain M5 and the putative hydrophobic transmembrane domain M4 in the model of subunit structure proposed by Finer-Moore and Stroud (1984). Anderson et al. (1983) observed that mAb 125 bound to a partially soluble 7000–8000-Da fragment of β subunits cleaved by trypsin from the extravesicular (intracellular) surface of nascent β subunits. This is consistent both with the mapping of mAb 125 on β subunits reported here and with the intracellular location of the putative M4 region on α subunits demonstrated in the following paper (Ratnam et al., 1986). Similarly, both immunochemical data (Figure 11) and immunoelectron microscopic data (Figure 12) clearly show that the binding site for mAb 125 is intracellular, which strongly suggests that the sequence β429–441 is intracellular. Since there is ample evidence that the C-termini of the receptor subunits are cytoplasmic (Figure 10; Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985), it follows that the proposed (Noda et al., 1983a; Finer-Moore & Stroud, 1984) transmembrane region M4 should in fact be cytoplasmic. It also follows that M5 is intracellular unless there is an additional transmembrane domain in the hydrophilic sequence between M3 and M5 in order to account for an odd number of total transmembrane domains required to keep the N- and C-termini on opposite sides of the membrane, and also the fact that some part of the sequence between M3 and M5 is intracellular.

To summarize the above results, mapping of the antigenic domains of the δ and β subunits strongly suggests the following features of the subunits in the native receptor structure: (1) the C-terminus is intracellular; (2) a region between M3 and M5 is intracellular; (3) the region between M5 and M4 is intracellular, implying that M4 and possibly M5 are not transmembrane domains as had previously been suggested (Claudio et al., 1983; Devilliers-Thiery et al., 1983; Noda et al., 1983a; Guy, 1983; Finer-Moore & Stroud, 1984). Our findings are confirmed by a different approach in our accom-

panying report in this issue (Ratnam et al., 1986), in which peptides having the sequences corresponding to the region between M3 and M4 in the α subunit were synthesized and the location of the binding sites of antibodies to these sequences was determined in the membrane-bound receptor.

Mapping mAbs That Affect Receptor Function. mAbs that bind to sites other than the agonist binding site of the receptor and affect its function should be useful probes for detecting other domains on the receptor which are closely associated with its function, especially the cation channel forming sequences. Hence, mapping of the binding sites of such mAbs is especially important. Among a large number of mAbs in our library, both against intact receptor and against denatured subunits (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1983, 1985a; S. Hochschwender et al., unpublished results), surprisingly few had any effect on receptor function (Wan & Lindstrom, 1985). mAbs against the MIR on the extracellular surface of the receptor and almost all of the mAbs against intracellular determinants had no effect on receptor function (Lindstrom et al., 1981b, 1983; Wan & Lindstrom, 1985; Blatt et al., 1986). mAb 148 (to β subunits) and mAb 168 (to γ subunits) were both shown to inhibit carbamylcholine-induced $^{22}Na^+$ uptake into reconstituted receptor vesicles without interfering with the process of reconstitution (Wan & Lindstrom, 1985) and also to inhibit channel activity in single-channel measurements of receptor reconstituted into lipid bilayers by the "patch clamp" method (Blatt et al., 1986). Since monovalent Fab fragments of these mAbs bound to receptor with little affect on its function, it was concluded that the primary functional effects of mAbs 148 and 168 were due to overall structural changes in the receptor rather than direct occlusion of the cation channel by the antibodies (Wan & Lindstrom, 1985). These mAbs were shown to produce their effect by cross-linking receptors on their intracellular surfaces and to alter the kinetics of binding of α-bungarotoxin to the agonist site on the extracellular surface of α subunits (Wan & Lindstrom, 1985). The binding site of mAb 148 was found in the present study to lie within the sequence 368–406 on the β subunit, i.e., similar to that of mAb 111 (Figures 3, 4, and 8 and Table II). However, mAb 111 bound to some large nonterminal β-subunit fragments to which mAb 148 did not (Figure 3), indicating that mAb 148 bound on the C-terminal side of mAb 111. It is difficult to interpret the functional effects of mAbs 10 and 13, which are obtained only at high mAb concentrations (Lindstrom et al., 1981b; Blatt et al., 1986), in terms of binding studies obtained with much lower mAb concentrations.

Mapping Antibody Binding Sites on α Subunits. Mapping of the antigenic structure of the α subunit is of special interest because of the role of this subunit in eliciting antibodies in the autoimmune disease myasthenia gravis (Lindstrom, 1985). Most of the antibodies against the native receptor both in the sera of rats immunized with the receptor (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1983) and in myasthenia gravis patient sera (Tzartos et al., 1982) are directed against the MIR, which is present on the extracellular surface of the α subunit (Tzartos & Lindstrom, 1980; Sargent et al., 1984). In fact, about 70% of the antigenic modulation of the receptor produced in cell cultures by patient sera is due to antibodies against the MIR (Tzartos et al., 1985b). Most of the antibodies against denatured subunits, on the other hand, bind to the intracellular surface of the receptor (Froehner, 1981; Anderson et al., 1983; Sargent et al., 1984). The mAbs against the denatured α subunit mapped by us in the present study were all directed against the C-terminal region of the α subunit. This observation also applies to our mapping data on the δ and β subunits discussed above. Thus, mAbs 8, 10, 13, 19, 61, 147, 149, 152, 153, 155, and 164 were mapped to the region α333–385 (Figures 6 and 10 and Table III). The antigenic determinant for mAb 149 has been located on the intracellular surface of the receptor by electron microscopy (Sargent et al., 1984). Similarly, the antigenic determinant for mAb 61 is accessible only in muscles which have been permeabilized by saponin (Sargent et al., 1984). These results are consistent with the results discussed earlier regarding the intracellular location of corresponding regions in the δ and β subunits. The positions of the binding domains of the mAbs mapped on the primary sequence of the α subunit are illustrated in Figure 8.

It was earlier proposed that the amino acid sequence α161–166 might represent the MIR, since this was the most hydrophilic sequence in the α subunit (Noda et al., 1982). This was disproved by the observations that mAbs to the MIR did not bind to the corresponding synthetic peptide (Juillerat et al., 1984; Lindstrom et al., 1984) and that antibodies to this sequence did not compete with anti-MIR mAbs for binding to the receptor (Lindstrom et al., 1984). In this study, the binding site of mAb 210 (a mAb to the MIR raised against receptor from mammalian muscle; S. Hochschwender et al., unpublished results) was mapped to the region α46–127 (Figures 7 and 8). The MIR is a conformation-dependent determinant, and antibodies against it have low affinity for the denatured α subunit but high affinity for native receptor. There is evidence that several closely spaced epitopes comprise the MIR [reviewed in Lindstrom (1983)]. Having bracketed the MIR to the sequence α46–127, it should be possible to precisely map it by testing the ability of mAbs to bind synthetic peptides corresponding to parts of this sequence.

Figure 9 shows the distribution of immunogenic determinants on the primary sequence of the α subunit, both for antibodies in an antiserum against the denatured α subunit and for antibodies in an antiserum to whole receptor. The antigenicity of the various parts of the α-subunit sequence was determined in terms of percent cross-reactivity of various synthetic peptides having these sequences with the antisera (Lindstrom et al., 1984; Criado et al., 1985a,b, 1986; Ratnam et al., 1986). Interestingly, the immunogenic determinants occur predominantly in a region close to the C-terminal end (α330–410), consistent with the mapping of the binding sites of most of our mAbs on this region. Most of the immunogenic regions in denatured α subunits (83%) have now been precisely mapped, whereas only 46% of the immunogenic regions in native α subunits have been precisely mapped because we do not yet have synthetic peptides corresponding to the MIR. There are some differences in the immunogenicity of certain sequences in native vs. denatured α subunits, but the pattern is relatively similar in both instances, and in most cases where a peptide does not react at all with antibodies to native receptor, it also does not react at all with antibodies to denatured α subunits. Fine-scale mapping of mAbs in our library using synthetic peptides in this region is described in the following paper (Ratnam et al., 1986).

Added in Proof

Souroujon et al. (1986) have recently reported finding a highly immunogenic region in the C-terminal 14-kDa portion of the α subunit. This corresponds to the highly immunogenic sequence, especially in denatured receptors, which we found in α, β, and δ subunits (Figures 8 and 9). Barkas et al. (1986) have recently reported mapping the MIR to the N terminus of α151, which is consistent with our mapping of the MIR to

between α46 and α127 (Figure 8). Lennon et al. (1985) have recently reported that α125–147 is exposed on the extracellular surface of receptor and can induce experimental autoimmune myasthenia gravis. These results are consistent with our observation that this sequence is a detectable immunogen in both native and denatured receptors (Figure 9), although our results (Criado et al., 1986) suggest that parts of this sequence are not clearly exposed. We also agree (Criado et al., 1986) that antibodies to this sequence do not compete with toxin for binding to receptor. However, Lennon et al. (1985) reported that α125–147 bound 26–56% of anti-receptor antibodies, implying that this peptide was the MIR. This is not correct. Figure 9 shows that many other parts of the sequence are much more immunogenic. Figure 2 from Lennon et al. (1985) clearly shows that the *maximum* antibody concentration against receptor which was achieved by immunization with peptide (6 nM) was less than 1% of the titer achieved by immunization with an equivalent weight of receptor! Their claim that 26–56% of antibodies bound to this sequence was based on an experiment [Table V of Lennon et al. (1985)] in which ^{125}I-labeled serum antibodies (in unspecified amounts) were added to Sepharose conjugates of unspecified amounts of either receptor or peptide, and then the number of counts bound to the conjugates was compared. This is meaningless. For example, if 1 nmol of receptor epitope was present in its conjugate, and 0.5 nmol of peptide epitope was present in its conjugate, then when excess antibody was added, the ratio of antibody bound to peptide conjugate/antibody bound to receptor conjugate would inevitably be 50%. The proper way to do the experiment would have been to adsorb antireceptor serum with the peptide conjugate and determine the maximum fraction of antibody titer which could be adsorbed. Finally, antibodies to α127–143 do not compete for binding to the receptor with antibodies to the MIR (Criado et al., 1986).

Acknowledgments

We thank John Cooper for purifying receptor and performing the radioiodinations. We thank Ru-Shya Liu for her major role in the preparation of mAbs to synthetic peptides. We thank Doug Schneider, J. Dyckert, and Diana Roberts for technical assistance and Maya Spies for typing the manuscript. We especially thank Robert Sealock for instructing P. B. Sargent on how to visualize antibody binding to receptor-rich vesicles using poly(vinyl chloride) wells and colloidal gold–protein A (Wray & Sealock, 1984) and for his generous gifts of pH 11 extracted receptor-rich membranes, of *Naja naja siamensis* toxin (cobra toxin), and affinity-purified rat anti-*N.n.s.* toxin.

References

Anderson, D. J., Walter, P., & Blobel, G. (1982) *J. Cell Biol. 93*, 501–506.

Anderson, D. J., Blobel, G., Tzartos, S., Gullick, W., & Lindstrom, J. (1983) *J. Neurosci. 3*, 1773–1784.

Barkas, T., Gabriel, J., Juillerat, M., Kokla, A., & Tzartos, S. (1986) *FEBS Lett.* (in press).

Bassiri, R., & Utiger, R. (1979) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M., & Behrman, H., Eds.) pp 46–47, Academic Press, New York.

Blatt, Y., Montal, M. S., Lindstrom, J., & Montal, M. (1986) *J. Neurosci. 6*, 481–486.

Bon, F. (1984) *J. Mol. Biol. 176*, 205–237.

Brisson, A., & Unwin, P. N. T. (1985) *Nature (London) 315*, 474–477.

Cartaud, J., Benedetti, E. L., Sobel, A., & Changeux, J.-P. (1978) *J. Cell Sci. 29*, 313–337.

Claudio, T., Ballivet, M., Patrick, J., & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1111–1115.

Criado, M., Hochschwender, S., Sarin, V., Fox, J. L., & Lindstrom, J. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2004–2008.

Criado, M., Sarin, V., Fox, J. L., & Lindstrom, J. (1985b) *Biochem. Biophys. Res. Commun. 128*, 864–871.

Criado, M., Sarin, V., Fox, J. L., & Lindstrom, J. (1986) *Biochemistry* (in press).

Devillers-Thiery, A., Giraudat, J., Bentaboulet, M., & Changeux, J.-P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2067–2071.

Elliott, J., Blanchard, S., Wu, W., Miller, J., Strader, C., Hartig, P. R., Moore, H.-P., Racs, J., & Raftery, M. (1980) *Biochem. J. 185*, 667–678.

Finer-Moore, J., & Stroud, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 155–159.

Froehner, S. C. (1981) *Biochemistry 20*, 4905–4915.

Froehner, S., Douville, K., Klink, S., & Culp, W. (1983) *J. Biol. Chem. 258*, 7112–7120.

Gullick, W., & Lindstrom, J. (1983) *Biochemistry 22*, 3801–3807.

Gullick, W., Tzartos, S., & Lindstrom, J. (1981) *Biochemistry 20*, 2173–2180.

Guy, R. (1983) *Biophys. J. 45*, 249–261.

Hochschwender, S., Langeberg, L. K., Schneider, D. W., & Lindstrom, J. M. (1985) in *Hybridomas in Biotechnology and Medicine* (Springer, T., Ed.) pp 223–238, Plenum Press, New York.

Horn, R., & Stevens, C. F. (1980) *Comments Mol. Cell. Biophys. 1*, 57–68.

Huang, L. Y. M., Catterall, W. A., & Ehrenstein, G. (1978) *J. Gen. Physiol. 71*, 397–410.

Juillerat, M., Barkas, T., & Tzartos, S. (1984) *FEBS Lett. 168*, 143–148.

Kao, P., Dwork, A., Kaldany, R., Silver, M., Wideman, J., Stein, S., & Karlin, A. (1984) *J. Biol. Chem. 259*, 11662–11665.

Kistler, J. K., & Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3678–3689.

Klymkowsky, M. W., & Stroud, R. M. (1979) *J. Mol. Biol. 128*, 319–334.

Kyte, J., & Rodriguez, H. (1983) *Anal. Biochem. 133*, 515–522.

La Rochelle, W. J., Wray, B. E., Sealock, R., & Froehner, S. C. (1985) *J. Cell Biol. 100*. 684–691.

Lennon, V., McCormick, D., Lambert, E., Griesmann, G., & Atassi, Z. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8805–8809.

Lewis, C. A. (1979) *J. Physiol. (London) 286*, 417–445.

Lewis, C. A., & Stevens, C. F. (1979) in *Membrane Transport Processes* (Stevens, C. F., & Tsien, R. W., Eds.) pp 133–157, Raven Press, New York.

Lindstrom, J. (1983) in *Monoclonal Antibodies: Probes For The Study Of Autoimmunity And Immunodeficiencies* (Eisenbarth, G. S., & Haynes, B. F., Eds.) pp 259–296, Academic Press, Orlando, FL.

Lindstrom, J. (1985) *Annu. Rev. Immunol. 3*, 109–131.

Lindstrom, J., Merlie, J., & Yogeeswaran, A. (1979a) *Biochemistry 18*, 4465–4470.

Lindstrom, J., Walter, B., & Einarson, B. (1979b) *Biochemistry 18*, 4470–4480.

Lindstrom, J., Einarson, B., & Tzartos, S. (1981a) *Methods Enzymol. 74*, 432–460.

Lindstrom, J., Tzartos, S., & Gullick, W. (1981b) *Ann. N.Y. Acad. Sci. 377*, 1–19.
Lindstrom, J., Criado, M., Hochschwender, S., Fox, J. L., & Sarin, V. (1984) *Nature (London) 311*, 573–575.
Mishina, M., Tobimatsu, T., Imoto, K., Tanaka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M., & Numa, S. (1985) *Nature (London) 313*, 364–369.
Muhlpfordt, H. (1982) *Experientia 38*, 1127–1128.
Neher, E., & Sakmann, B. (1976) *Nature (London) 260*, 799–802.
Neubig, R., Krodel, E., Boyd, N., & Cohen, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.
Noda, M., Takahashi, H., Tanabe, T., Yoyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature (London) 299*, 793–797.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983a) *Nature (London) 302*, 528–532.
Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kanayo, T., Hirose, T., Inayama, S., & Numa, S. (1983b) *Nature (London) 305*, 818–823.
Raftery, M., Hunkapillar, M., Strader, C., & Hood, L. (1980) *Science (Washington, D.C.) 208*, 1454–1457.
Ratnam, M., & Lindstrom, J. (1984) *Biochem. Biophys. Res. Commun. 122*, 1225–1233.
Ratnam, M., Le Nguyen, D., Rivier, J., Sargent, P., & Lindstrom, J. (1986a) *Biochemistry* (following paper in this issue).
Ratnam, M., Gullick, B., Spiess, J., Wan, K., Criado, M., & Lindstrom, J. (1986b) *Biochemistry* (in press).
Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.
Sargent, P. B., Hedges, B. E., Travaler, L., Clemmons, L., Tzartos, S., & Lindstrom, J. M. (1984) *J. Cell Biol. 98*, 609–618.
Sealock, R. (1982) *J. Cell Biol. 92*, 514–522.
Seed, B. (1982) *Nucleic Acids Res. 10*, 1799–1810.
Slot, J. W., & Geuze, H. V. (1981) *J. Cell Biol. 90*, 533–536.
Sobel, A., Weber, M., & Changeux, J.-P. (1977) *Eur. J. Biochem. 80*, 215–224.
Souroujon, M., Neumann, D., Pizzighella, Safran, A., & Fuchs, S. (1986) *Biochem. Biophys. Res. Commun. 135*, 82–89.
Steinbach, J. H., & Stevens, C. F. (1976) in *Neurobiology of the Frog* (Llinas, R., & Precht, W., Eds.) pp 33–92, Springer-Verlag, West Berlin.
Sumikawa, K., Houghton, M., Smith, J., Bell, L., Richards, B., & Barnard, E. (1982) *Nucleic Acids Res. 10*. 5809–5822.
Tzartos, S. J., & Lindstrom, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.
Tzartos, S. J., Rand, D. E., Einarson, B. L., & Lindstrom, J. M. (1981) *J. Biol. Chem. 256*, 8635–8645.
Tzartos, S. J., Seybold, M., & Lindstrom, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 188.
Tzartos, S. J., Langeberg, L., Hochschwender, S., & Lindstrom, J. (1983) *FEBS Lett. 158*, 116–118.
Tzartos, S. J., Langeberg, L., Hochschwender, S., Swanson, L. W., & Lindstrom, J. (1985a) *J. Neuroimmunol. 10*, 235–253.
Tzartos, S. J., Sophianos, D., & Efthimiadis, A. (1985b) *J. Immunol. 134*, 2343–2349.
Wan, K. K., & Lindstrom, J. M. (1985) *Biochemistry 24*, 1212–1221.
Wise, D., Karlin, A., & Schoenborn, B. P. (1979) *Biophys. J. 28*, 473–496.
Wray, B. E., & Sealock, R. (1984) *J. Histochem. Cytochem. 31*, 1117–1120.
Young, E. F., Ralston, E., Blake, J., Ramachandran, J., Hall, Z. W., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626–630.
Zingsheim, H. P., Neugebauer, D.-C., Barrantes, F. J., & Frank, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 952–956.