Immunochemical Demonstration That Amino Acids 360-377 of the Acetylcholine Receptor Gamma-Subunit Are Cytoplasmic

WILLIAM J. LAROCHELLE,* BARNABY E. WRAY,[‡] ROBERT SEALOCK,[‡] and STANLEY C. FROEHNER*
*Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756; and [‡]Department of Physiology and The Neurobiology Program, University of North Carolina, Chapel Hill, North Carolina 27514

ABSTRACT Two monoclonal antibodies (mabs) previously prepared against *Torpedo* acetylcholine receptor are shown to recognize a synthetic nonadecapeptide corresponding to lys_{360}-glu_{377} of the gamma subunit. The reaction was demonstrated by solid-phase enzyme-linked immunoabsorbent assays, by inhibition of binding of the mabs to receptor, and by immunoprecipitation of the peptide conjugated to bovine serum albumin. Immunogold electron microscopy on isolated postsynaptic membranes from *Torpedo* showed that both mabs bind to intracellular epitopes on the receptor. These results establish that amino acid residues 360-377 of the receptor gamma-subunit, and probably the analogous region of the delta-subunit, reside on the cytoplasmic side of the membrane. Since the primary structures of all four subunits suggest a common transmembrane arrangement, the corresponding domains of the alpha- and beta-subunits are probably also cytoplasmic.

The recent identification of the amino acid sequences of all four subunits of the acetylcholine receptor (AChR)[1] from *Torpedo* electric organ (5, 7, 24, 29, 36) has led to models for the transmembrane folding pattern of the subunits and for the structure of the receptor's ion channel (for a review, see reference 27). The results of these studies are likely to be of wide impact, because the receptors from *Torpedo* and mammalian muscle share extensive homology (6, 21, 25) and because they may provide a theoretical framework for the structure and function of other neurotransmitter receptors. Thus, prompt direct tests of these models are of major importance.

The polypeptide chain of each receptor subunit contains a large hydrophilic domain at the N-terminus followed by three closely spaced hydrophobic regions, a second large hydrophilic domain, and a fourth hydrophobic domain (5, 7, 26). The recent identification of cysteine 192 as part of the alpha-chain that contributes to the acetylcholine-binding site (41) and evidence from cell-free synthesis of receptor subunits (1) establish that the large N-terminal domain is extracellular. In folding models based on hydrophobicity analyses alone, the hydrophobic domains form the only transmembrane elements, probably as helices. Hence, these models predict that the second hydrophilic domain lies on the cytoplasmic side of the membrane and that the C-terminus is extracellular (5, 7, 26). Searches of the sequences for repeating arrangements of nonpolar and polar residues, however, have produced evidence for an additional, amphipathic helix just preceeding the fourth hydrophobic helix (9, 17). Thus, in this class of model, the large cytoplasmic domain is smaller and the C-terminus is cytoplasmic. In a third model, the second hydrophilic domain is divided roughly into two halves joined by a transmembrane element (19).

The most direct initial tests of the proposed folding patterns require localization of identified regions of the amino acid sequences relative to the membrane bilayer. Antibodies raised against synthetic peptides that correspond to selected receptor polypeptide sequences or existing antibodies whose epitopes

[1] *Abbreviations used in this paper:* AChR, nicotinic acetylcholine receptor; ELISA, enzyme-linked immunoabsorbent assay; KLH, keyhole limpet hemocyanin; mab, monoclonal antibody.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 MARCH 1985 684-691
© The Rockefeller University Press · 0021-9525/85/03/0684/08 $1.00

can be identified will undoubtedly be major tools in these experiments. Thus, peptides corresponding to the C-terminal sequences have been used to screen a monoclonal library and as immunogens for the production of antibodies (42, 43). The resulting antibodies recognize cytoplasmic epitopes on the membrane-bound receptor (42, 43), supporting the existence of an amphipathic transmembrane region. In this paper, we show that two monoclonal antibodies (mabs) raised against denatured AChR recognize a synthetic peptide corresponding to amino acid residues 360-377 of the gamma subunit. This region lies within the second hydrophilic domain, and has been predicted to be exposed to the cytoplasm (5, 7, 9, 17, 26) or embedded in the membrane (19). Using ultrastructural immunocytochemistry, we show that these mabs react with exposed cytoplasmic epitopes, and that these epitopes are not involved in binding of the receptor-associated M_r 43,000 protein.

MATERIALS AND METHODS

Antibodies: The production and characterization of the mabs to *Torpedo californica* AChR and the purification of IgG from ascites fluids have been described (13). Rabbit antitoxin antibodies (IgG) were raised against hemocyanin-coupled α-neurotoxin III from *Naja naja* siamensis venom (18), isolated by ammonium sulfate precipitation and ion exchange chromatography (15), and affinity-purified by standard procedures.

Synthetic Peptides: The nonadecapeptide that corresponds to amino acid sequence 360-377 of the AChR gamma subunit (plus a cysteine on the carboxyl terminus) was synthesized by Sequemat Inc. (Watertown, MA). The peptide was judged to be homogeneous by high performance liquid chromatography performed by the supplier and by fast protein liquid chromatography ion exchange chromatography using a Pharmacia Mono Q column (Pharmacia Fine Chemicals, Piscataway, NJ). The sequence was verified by the supplier. Disulfide-linked dimer of the peptide was formed by incubation of peptide dissolved at 1 mM in 10 mM sodium phosphate, pH 8.0, 0.02% NaN_3 for 40 h at room temperature. Less than 0.2% of the sulfhydryl groups remained in the reduced form after this treatment. The hexadecapeptide corresponding to residues 485-499 of the delta chain and its bovine serum albumin (BSA) conjugate were kindly provided by Dr. Robert Stroud's laboratory (University of California at San Francisco). This peptide has the sequence Ac-Pro-Phe-Glu-Gly-Asp-Pro-Phe-Asp-Tyr-Ser-Ser-Asp-His-Pro-Arg-Gly-OH.

Preparation of the Peptide Conjugate: A modification of the procedures described by Gentry et al. (16) for attachment of peptides via sulfhydryl groups to carrier proteins was used. All procedures were performed at 25°C. BSA (4 mg dissolved in 250 μl of 10 mM sodium phosphate, pH 7.4) was alkylated with 250 nmol of *N*-ethylmaleimide for 10 min and then ten 10-μl aliquots of maleimidobenzoyl-*N*-hydroxysuccinimide ester (20 mg/ml in dimethylformamide) was added. After 30 min, the sample was passed through a 0.8 × 18 cm BioGel P60 column equilibrated with 50 mM sodium phosphate buffer, pH 6.0, to remove excess alkylating agent and maleimidobenzoyl-*N*-hydroxysuccinimide ester. Fractions corresponding to the void volume were pooled and adjusted to pH 7.4 with 1 N NaOH. This sample was then divided into two parts and one was mixed with 1.5 mg gamma-peptide 360-377 (dissolved in 200 μl 10 mM sodium phosphate, pH 7.4). The other part was mixed with buffer and served as control BSA. After incubation for 4 h, the conjugate was separated from the free peptide on a Sephadex G-50 column equilibrated in 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS). Comparison of the amino acid compositions of peptide-BSA conjugate and control BSA showed that ~7 mol of peptide were conjugated per mol of BSA. The keynole limpet hemocyanin (KLH) conjugate was prepared in a similar manner.

Radioiodination of Peptide-BSA Conjugate: Peptide-BSA conjugate (125 μg) or control BSA (125 μg) was incubated with 0.1 nmol $Na^{125}I$ (200 μCi) and 225 nmol chloramine T in 65 μl of 0.1 M $NaPO_4$, pH 7.4, for 1 min on ice. The reaction was stopped by the addition of 5 μmol of dithiothreitol and the iodinated protein was separated from sodium iodide by chromatography on a BioGel P60 column equilibrated with PBS. Specific radioactivities of 26 μCi/nmol for the conjugate and 20 μCi/nmol for control BSA were obtained.

Enzyme-linked Immunoadsorption Assay: Microtiter wells of a Falcon flexible assay plate (3912) were incubated with peptide-BSA conjugate or control BSA (300 ng in 50 μl of PBS, 0.02% azide) for 2 h. The solutions were removed, the wells were incubated with PBS/NaN_3 containing 4% BSA for 30 min, and the plates were then washed with PBS/NaN_3 containing 0.05% Tween. All subsequent washes were done with the same solution. The wells were then incubated with 50 μl mab IgG (diluted in PBS/NaN_3/Tween containing 1% BSA) for 2 h, washed, and then incubated with antimouse immunoglobulin conjugated to β-galactosidase (BRL Scientific, Gaithersburg, MD) (1:100) for 3 h. The wells were washed again and incubated with para-nitrophenyl-β-D-galactoside (1 mg/ml) dissolved in 50 mM sodium phosphate buffer, pH 7.2, 1.5 mM $MgCl_2$, and 100 mM 2-mercaptoethanol. The reaction was quenched with 0.5 M sodium carbonate and absorbance was read at 405 nm.

For the inhibition of binding experiments, mabs were incubated with the inhibitor (peptides, peptide-BSA conjugate, control BSA) for 1 h and then transferred to wells coated with 500 ng purified *Torpedo nobiliana* AChR (12). After incubation for times determined in separate experiments to give 50–70% maximal binding of mabs, the plates were processed as described above. The following IgG concentrations were used: 10 nM for 88B and 147A; 20 nM for 264E, 139A, 240A, 245A, 262C, and 280B; 40 nM for 274D.

Immunoprecipitation of Radioiodinated Peptide-BSA Conjugate: Mab IgG (10 μl) was incubated with 10 μl of ^{125}I-peptide conjugate or control BSA (250 fmol) for 60 min. Immunoprecipitation was then performed with fixed *Staphylococcus aureus* as previously described (13) with the following modification. Complexes of anti-mouse IgG and *S. aureus* were preformed by incubation of 150 μl of rabbit anti-mouse IgG serum with 1 ml of IgGsorb (10%; The Enzyme Center, Boston, MA) for 30 min at 37°C, washed to remove unbound antibody, and resuspended in the original volume. 30 μl was added to each reaction mixture to precipitate the mabs.

Preparation of Membrane Fragments: Partially purified AChR-rich membrane fragments were prepared from frozen *T. californica* electric tissue by two procedures. (*a*) Extracted membranes: Membranes were prepared according to Sobel et al. (35), except that 5 mM EDTA was added to the first supernatant to retard proteolysis and the final, linear sucrose gradient was omitted. The membranes in the receptor-rich fraction of the discontinuous gradient were pelleted, resuspended in water, adjusted to pH 11 with 1 M NaOH, sonicated for 0.5–1 min at room temperature in a small bath-type sonicator (Sonicor Instrument Corp., Copiague, NY), pelleted, resuspended in water to their initial volume, and stored frozen in 50-μl aliquots. (*b*) Nonextracted membranes: Tissue was homogenized in 25% sucrose–2 mM $ZnCl_2$ and fractionation by differential and sucrose gradient centrifugation (unpublished results; 34) to obtain a crude fraction of membranes in the form of open sheets and tubes. These membranes were prepared shortly before use.

Colloidal Gold Particles: Colloidal gold was prepared using sodium citrate (15-nm gold particles; 10, 31) or sodium citrate/tannic acid (5-nm particles; 22) as the reducing agent. Each size was complexed with *S. aureus* protein A (Pharmacia Fine Chemicals) by standard methods (e.g., see reference 31) as modified by Wray and Sealock (39). The large and small gold particles were used for labeling at absorbances of 2.0 at 520 nm and 1.0 at 505 nm, respectively.

Labeling Procedure: Membrane fragments were labeled with 0.1 μM mab 264E IgG or 0.2 μM mab 274D IgG followed by rabbit antimouse IgG serum diluted 1:50 and protein A–5 nm colloidal gold. They were then labeled with 1 μM α-toxin, 1 μM antitoxin IgG, and protein A–15 nm colloidal gold. These procedures were performed using polylysine-coated microculture wells as described by Wray and Sealock (39) with some modifications. After application to well bottoms by centrifugation, the membranes were fixed with 0.2% glutaraldehyde (electron microscopy grade, Polysciences, Inc., Warrington, PA) for 30 min, then treated with $NaBH_4$ (1 mg/ml, 30 min) to reduce aldehydes and Schiff's bases (38) and possibly to restore antigenicity (8). This fixation/reduction step was repeated between the incubations with antireceptor mabs and rabbit antimouse IgG. A third fixation (1% glutaraldehyde) and reduction was given after application of protein A–5 nm colloidal gold. Preliminary tests established that these fixations did not diminish the effectiveness of the immunochemical steps. After application of protein A–15 nm colloidal gold, the membranes were fixed with 1.6% glutaraldehyde-tannic acid in PBS at pH 7.3, and prepared for electron microscopy as described (39).

In competition experiments, mabs were diluted to their final concentration in solutions of peptide dimer at various concentrations. The solutions were incubated for 30 min at room temperature and centrifuged for 20 min at 20,000 *g*.

Receptor-rich membrane fragments were identified by the presence of labeling on the α-neurotoxin. Within aggregations of membranes, fragments were often labeled by antireceptor mabs but not by the α-toxin, certainly because of hindrance of access of reagents to the outer surfaces of the fragments (see Fig. 6 *A*). These fragments were excluded from all analyses. Three experiments gave identical qualitative results and one was selected for quantitative analysis. Every receptor-rich fragment or portion thereof for which there was no obvious impediment to labeling (such as adhesion to an adjacent fragment) was identi-

fied in data micrographs. The total numbers of small and large gold particles on the inner and outer surfaces, respectively, were counted and the ratio was taken. Up to 300 fragments and from 5,000 to 8,000 large gold particles (extracted membranes) or 1,200 large gold particles (nonextracted membranes) were counted for each sample.

RESULTS

Selection of the Synthetic Peptide

In previous work, we generated a panel of monoclonal antibodies against the AChR from *Torpedo* (13). Four of these mabs (88B, 147A, 264E, and 274D) were found to react with both the gamma- and delta-subunits of the receptor on immunoblots. (Mab 264E was originally reported to recognize only the delta-subunit. Recently, however, we have found that it also recognizes the gamma-subunit under certain conditions of immunoblotting [unpublished results].) Binding studies with isolated membranes suggested that these mabs recognize cytoplasmic determinants. Therefore, we sought to identify a proposed cytoplasmic sequence that was hydrophilic, was likely to be antigenic, and had considerable homology between the delta- and gamma-subunits while sharing little homology with the other subunits. This led to the selection of gamma-peptide 360-377, the sequence of which is shown in Fig. 1, as a candidate for the binding site for one or more of the anti-AChR mabs. (Numerical assignments of amino acid positions begin with the first residue of each mature subunit and continue without gaps.) The peptide shares 67% homology (12 of 18 residues identical) with the corresponding region (residues 368-385) of the delta-subunit (Fig. 1). Comparison of all tripeptide sequences present in the synthetic peptide with the entire sequences of each of the subunits shows the following identities: Ala-Glu-Glu, alpha 395-397 and gamma 162-164; Leu-Lys-Lys, beta 424-426; Leu-Met-Phe, beta 284-286 and delta 292-294; Ser-Glu-Leu, gamma 82-84. No sequence identities longer than tripeptides were found. Since antigenic determinants are comprised of a sequence of six or seven residues (2), antibodies directed against this peptide are highly unlikely to recognize epitopes located outside the region 360-377 of the gamma-subunit and the homologous region of the delta-chain.

Mabs 264E and 274D Recognize Gamma-Peptide 360-377

The set of AChR mabs was screened for reactivity with gamma-peptide 360-377 conjugated to BSA and KLH using a solid-phase enzyme-linked immunoabsorbent assay (ELISA). Mabs 274D and 264E were found to bind the conjugates; they did not bind BSA or KLH that had been treated with cross-linker in the absence of the peptide (Fig. 2). Nine other mabs, including ones that recognize the AChR delta-subunit (240A, 245A, 262C, 280B), the gamma- and the delta-subunits (88B, 147A), and the alpha-subunit (139A) and two control mabs that do not recognize the receptor (MPC-11, MOPC-21) were unreactive with either peptide-BSA or peptide-KLH. None of the anti-AChR mabs reacted with a BSA conjugate of a synthetic peptide corresponding to residues 485-499 of the delta subunit (data not shown).

PEPTIDE	Lys-Ala-Glu-Glu-Tyr-Ile-Leu-Lys-Lys-Pro-Arg-Ser-Glu-Leu-Met-Phe-Glu-Glu-Cys
GAMMA SUBUNIT	Lys-Ala-Glu-Glu-Tyr-Ile-Leu-Lys-Lys-Pro-Arg-Ser-Glu-Leu-Met-Phe-Glu-Glu 360 377
DELTA SUBUNIT	Lys-Ala-Gln-Glu-Tyr-Phe-Asn-Ile-Lys-Ser-Arg-Ser-Glu-Leu-Met-Phe-Glu-Lys 368 385

FIGURE 1 Comparison of the synthetic peptide sequence, gamma-subunit sequence 360–377, and delta-subunit sequence 368–385.

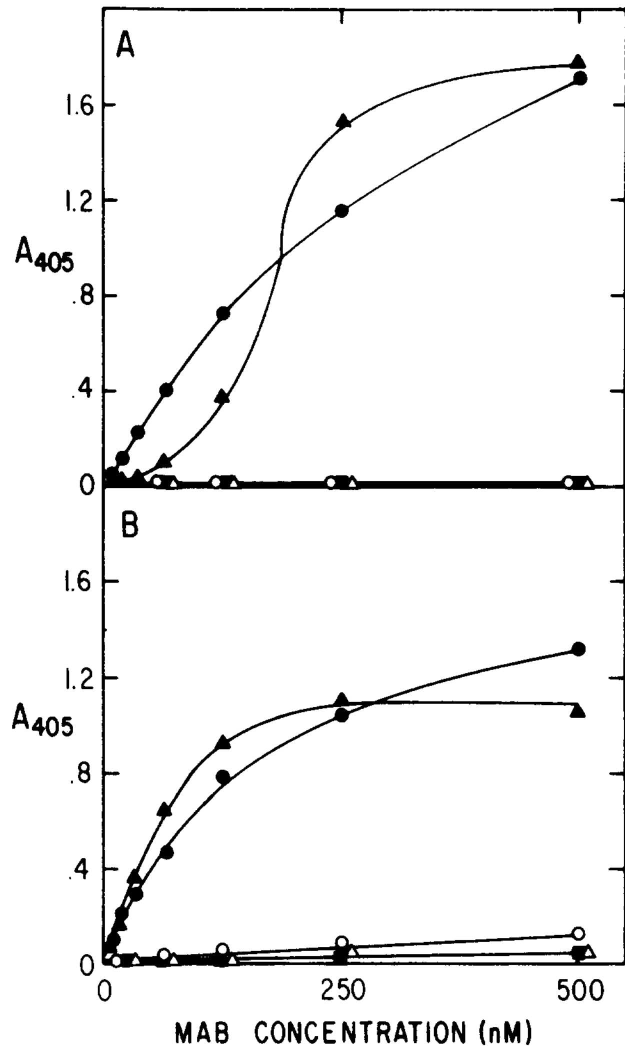

FIGURE 2 Solid-phase ELISA screening of mab binding to peptide conjugate. Binding of mabs 274D (▲, △), 264E (● ○), and 139A (■, □) was measured as described in Materials and Methods. (*A*) Microtiter wells were coated with BSA-peptide conjugate (closed symbols) or control BSA (open symbols). (*B*) Wells were coated with KLH-peptide conjugate (closed symbols) or control KLH (open symbols).

Gamma-peptide 360-377 effectively inhibited the binding of mabs 264E and 274D to AChR. In an ELISA in which mabs were preincubated with various concentrations of peptide before being added to wells coated with AChR, the peptide in its disulfide-linked dimeric form inhibited the rate of binding of mab 264E and mab 274D half-maximally at 6 μM and 30 nM, respectively (Fig. 3). Reduced, monomeric gamma peptide 360-377 also inhibited binding of mabs 264E and 274D but concentrations approximately threefold higher were necessary to achieve half-maximal inhibition (data not shown). Delta-peptide 485-499, tested at concentrations exceeding 36 μM, failed to inhibit the binding of either mab.

The apparent affinity of both mabs for gamma-peptide 360-377 was increased by conjugation of the peptide to BSA. Peptide-BSA conjugate competed half-maximally with AChR for mabs 264E and 274D at peptide concentrations of 600 and 2 nM, respectively (Fig. 4). BSA treated with cross-linker in the absence of peptide failed to inhibit the binding of 264E

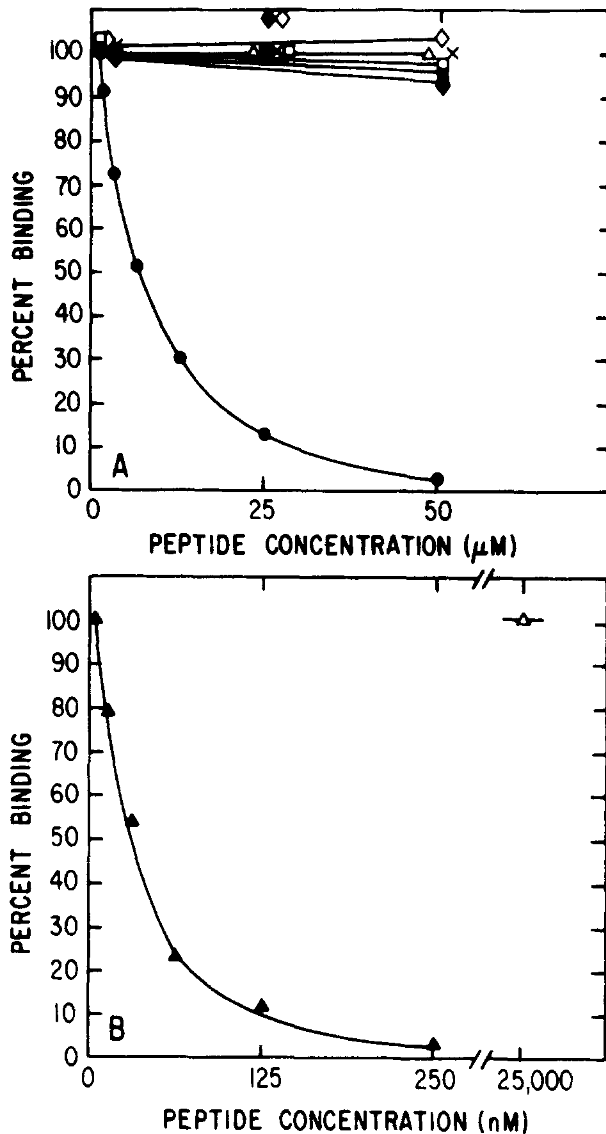

FIGURE 3 Inhibition of binding of mabs to AChR by peptides. (*A*) Mabs 264E (●), 139A (■), 88B (♦), 147A (◇), 240A (x), or 280B (□) were incubated for 1 h with dimeric gamma peptide 360–377. Mab 264E was also incubated with delta-peptide 485–499 (△). The samples were then transferred to microtiter wells coated with AChR and mab binding was measured after 15 min as described in Materials and Methods. (*B*) Mab 274D was incubated with gamma-peptide 360–377 (▲) or delta peptide 485–499 (△) for 1 h and then transferred to wells coated with AChR. Mab binding was measured after 30 min.

or 274D. Neither free peptide nor peptide-BSA conjugate inhibited the binding to receptor of the other seven mabs.

Mabs 264E and 274D also immunoprecipitated ^{125}I-peptide-BSA conjugate but not BSA that had been treated with cross-linker in the absence of peptide and then iodinated (Fig. 5). The seven other anti-AChR mabs and mabs MPC-11 and MOPC-21 failed to immunoprecipitate iodinated conjugate. Since radioiodination was performed after conjugation of peptide to BSA, most of the iodine-125 was probably incorporated into tyrosine residues of BSA. Attempts to immunoprecipitate ^{125}I-peptide were less successful, possibly because iodination alters the epitope(s) recognized by mabs 264E and 274D (data not shown).

Ultrastructural Localization of mab Binding Sites

Binding sites for mabs 264E and 274D were localized with respect to the lipid bilayer in extracted and nonextracted postsynaptic membranes from electric tissue. The former were receptor-rich membrane fragments from which peripheral membrane proteins, which could possibly block access of the antibodies to the receptor, had been removed by alkaline extraction (23). They were also sonicated to ensure that the majority was open or unsealed. Gel electrophoresis in the presence of SDS confirmed that substantial amounts of protein, including virtually all of the receptor-specific 43,000-mol-wt protein (23, 28, 35) had been removed. The nonextracted membranes were open fragments that retain substantial amounts of postsynaptic-specific submembrane material

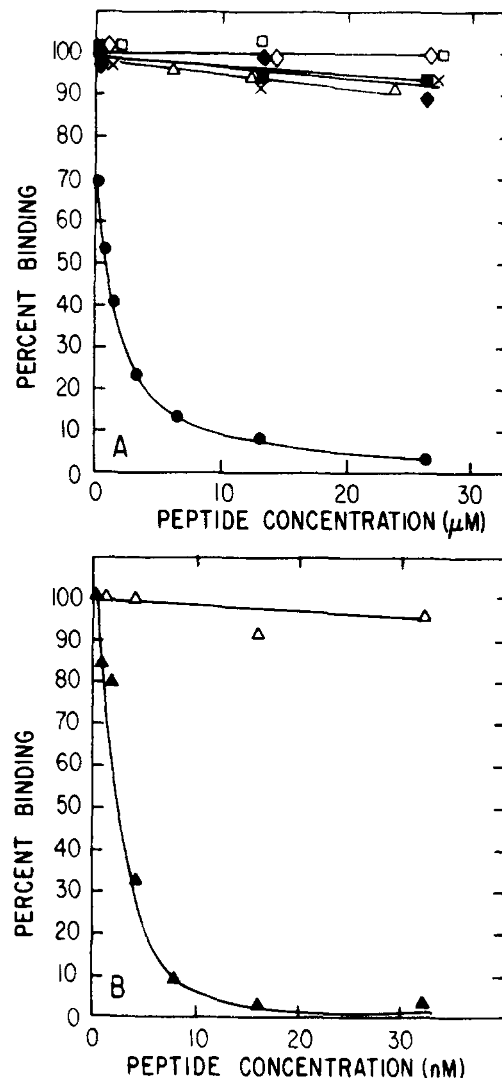

FIGURE 4 Inhibition of binding of mabs to AChR by peptide-BSA conjugate. (*A*) Mabs 264E (●), 139A (■), 88B (♦), 147A (◇), 240A (x), 245A (▼), 262C (▽), or 280B (□) were incubated for 1 h with peptide-BSA conjugate. Mab 264E was also incubated with control BSA (△). The samples were transferred to microtiter wells coated with AChR and mab binding was measured after 15 min as described in Materials and Methods. (*B*) Mab 274D was incubated for 1 h with peptide-BSA conjugate (▲) or control BSA (△) and the samples were transferred to wells coated with AChR. Mab binding was measured after 30 min.

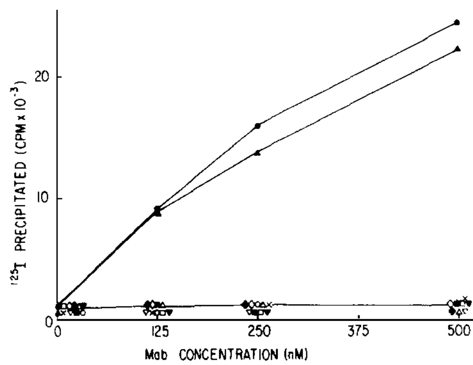

FIGURE 5 Immunoprecipitation of ^{125}I-peptide-BSA conjugate. Mabs 264E (●), 274D (▲), 139A (■), 88B (♦), 147A (◇), 240A (x), 245A (▼), 262C (▽), or 280B (□) and 274D (△) were also incubated for the same time period with iodinated control BSA. Immunoprecipitation was measured as described in Materials and Methods.

(R. Sealock, unpublished results), including the 43,000-mol-wt protein (34). All samples were lightly fixed before labeling to prevent loss of material and spontaneous resealing. Approximately 90% of the postsynaptic membranes remained open during the labeling procedures.

Mabs 264E and 274D gave similar qualitative patterns of labeling on both extracted membranes (Fig. 6 *A–D*; small gold particles) and nonextracted membranes (Fig. 6, *E* and *F*). Approximately 99% of the small gold particles (1,721 out of 1,736 counted) in labeled regions were on the cytoplasmic membrane surface, distributed essentially coextensively with the extracellular label on receptor-bound α-toxin (Fig. 6, *A* and *B*; large gold particles). On the basis of these results, we conclude that mabs 264E and 274D bind specifically to cytoplasmic determinants on the acetylcholine receptor.

Several findings provide additional evidence for the specificity of mab binding. Neither nonspecific mouse IgG (0.1 μM; Fig. 6*I*) nor any of four anti-43,000-mol-wt mabs (34) (not shown) bound to a significant degree to the extracted membranes. Incubation of the mabs with gamma-peptide 360-377 before their use reduced labeling due to mab 264E by 57% (100-μM peptide) and that due to mab 274D by 82% (1-μM peptide) (Table I). These extents of inhibition are lower than might be expected from the inhibition achieved with ELISA (Fig. 3). However, the concentrations of the mabs were fivefold higher and the incubations times were longer in the immunocytochemical experiments. Both of these conditions favor increased reaction with the receptor since the mabs have higher affinities for the receptor than for the peptide. Finally, the extracellular label was specific for the receptor since omission of the toxin completely abolished labeling by antitoxin IgG (Fig. 6*G*), and addition of 10 mM carbamylcholine (a competitive inhibitor of toxin binding to the receptor) to solutions of the toxin substantially reduced the labeling (Fig. 6*H*). The explanation for the failure of carbamylcholine to completely abolish toxin binding appears to be analogous to that for peptide inhibition of binding of mab 264E.

In nonextracted membranes, the two mabs gave quantitatively similar results (Table I). In extracted membranes, however, some quantitative differences were noted. Most receptor-rich regions were labeled strongly by mab 264E, the average region containing almost two small gold particles for every large particle (Table I). Mab 274D (0.2 μM) also often gave strong labeling (Fig. 6*B*), but generally labeled substantially less well than mab 264E (approximately 0.6 small particles/large particle, Table I; Fig. 6, *C* and *D*). The labeling was not significantly strengthened by increasing the concentration of mab 274D to 1 μM, or by shortening the wash between application of the antibody and the subsequent fixation to only 3 min. Mab 274D also labels less well than mab 264E if protein A–colloidal gold is applied directly to the mabs without rabbit antimouse IgG (unpublished results). One explanation for these results is that mab 274D may recognize only the gamma-subunit of the receptor in extracted membranes while 264E may recognize both the gamma- and delta-chains.

DISCUSSION

In this study, we have shown that two mabs that recognize the gamma- and delta-subunits of the AChR also recognize the gamma peptide 360-377, while seven other mabs against the receptor and two control mabs do not. Moreover, of the two reactive antibodies, mab 274D, which reacts preferentially with the gamma subunit, has the higher affinity for the peptide; mab 264E, which reacts more strongly with the delta-subunit, has a substantially lower affinity for the peptide. These observations and the relatively high affinities of both mabs for the peptide suggest that the reaction is highly specific. Thus, mabs 264E and 274D can be expected to react, in whole or in part, with the sequence 360-377 in the gamma-subunit of the intact receptor, and with the homologous region in the delta-subunit, since no identical sequence longer than a tripeptide is found outside these regions in the same or other subunits. Our results thus establish that amino acid residues 360-377 of the receptor gamma-subunit (and probably the homologous region of the delta chain) reside on the cytoplasmic side of the membrane.

Since the N-termini of the receptor subunits reside on the extracellular side of the postsynaptic membrane (1, 41), our results require an uneven number of membrane crossings between the N-terminus and the epitopes recognized by mabs 264E and 274D. They thus support models for the gamma- and delta-subunits in which three hydrophobic, transmembrane helices separate these two regions (5, 7, 9, 17, 26). These models place the region gamma 360-377 in the largest of the proposed cytoplasmic domains, which begins near residue 314 and ends when the polypeptide chain reenters the lipid bilayer, either in the region of tyr-403 (in the five helix models of Finer-Moore and Stroud [9] and of Guy [17]) or near val 467 (if only four transmembrane helices exist). The corresponding regions of the alpha- and beta-subunits are probably also cytoplasmic since the conserved primary structure of all four subunits suggests a common transmembrane arrangement (9, 26). The strong reactivity of the antibodies with this region further suggests that it is readily accessible. Hence, it presumably lies near the surface of the domain; it is unlikely to be sandwiched between the rest of the domain and the phosphate head groups of the membrane, for example.

One model proposes that amino acids 360-377 of the gamma-subunit lie within the bilayer, forming one wall of the receptor's ion channel (19). Our results appear to be incompatible with this hypothesis.

The antigen used for the production of mabs 264E and 274D was affinity-purified AChR denatured with SDS before injection. We have shown previously that polyclonal antibodies elicited by injection of denatured AChR into rabbits were

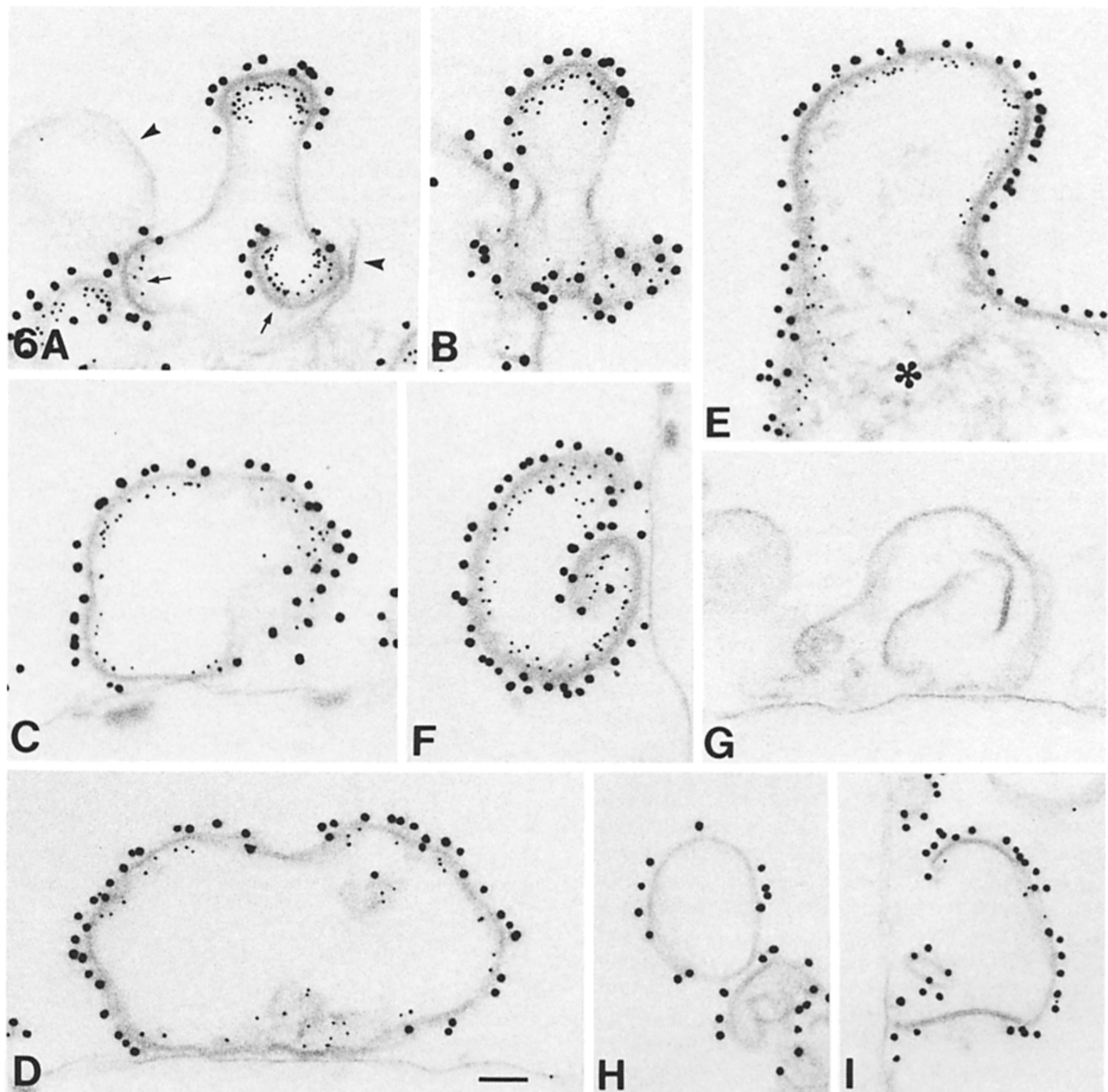

FIGURE 6 Localization of mab binding sites on postsynaptic membranes. Large gold particles: α-toxin and antitoxin IgG. Small gold particles: antireceptor mabs. (*A–D* and *G–I*) Extracted membranes. (*E* and *F*) Nonextracted membranes. (*A*) Labeled with mab 264E. Some receptor-rich regions are not labeled by the α-toxin due to steric block by adjacent membrane fragments (arrows). Contaminating nonpostsynaptic membranes are not labeled (arrowheads). (*B–D*) Labeled with mab 274D. The antibody sometimes labeled strongly (*B*), but it generally labeled more weakly (*C* and *D*) than mab 264E. (*E*) Labeled with mab 264E. This nonextracted membrane retained associated cytoplasmic filaments (*). (*F*) Labeled with mab 274D. (*G*) Labeled with antitoxin, but without α-toxin or mabs. The membranes shown are probably postsynaptic membranes, as judged by their morphology. They are typical of the sample. (*H*) Exposed to α-toxin in the presence of 10 mM carbamylcholine, then labeled with antitoxin IgG. Labeling was decreased but not abolished. (*I*) Labeled with mouse control IgG in place of mabs. Background labeling on the cytoplasmic surface of the membrane shown (four small gold particles) is higher than on most membranes in the sample. Bar, 100 nm (*A–D* and *F–H*) or 115 nm (*E* and *I*). (*A–D* and *F–H*; × 76,000). (*E* and *I*; × 66,000).

directed primarily against cytoplasmic determinants (11). Similar results were obtained in the production of monoclonal antibodies (13, 32). Mabs 88B and 147A (both of which recognize the gamma- and delta-subunits) compete with each other and with mab 264E in binding to native AChR (S. C. Froehner, unpublished results), suggesting that they recognize epitopes that lie close to each other. Thus, four out of fourteen mabs raised against AChR appear to be directed against a relatively small, highly antigenic sequence that possesses considerable homology between the gamma- and delta-chains. Predictions of the secondary structure of the region encompassing residues 360-377 of the gamma-chain suggest that it lacks both alpha-helix and beta-sheet structure (9). It is intriguing that an extracellular region of the alpha-chain (residues 161-166) that has been proposed (26) as a candidate for the "main immunogenic region" (37) is also predicted to lack these common ordered structures.

Mabs 264E, 88B, and 147A have previously been shown to react with the receptor in frog muscle in immunofluorescence experiments, but only if the antibodies had access to the muscle cytoplasm (13). Mabs 88B and 147A also react with rat muscle (13). These results predict that muscle receptors

TABLE I
Binding of Antireceptor mabs to Postsynaptic Membranes

	Ratio	Ratio (+ peptide)/ Ratio (− peptide)
Extracted membranes		
264E	1.69	0.43
264E + peptide (100 μM)	0.72	
274D	0.56	0.18
274D + peptide (1 μM)	0.10	
Nonextracted membranes		
264E	0.81	
274D	1.09	

The signal for bound antireceptor mabs is expressed relative to that for bound α-toxin: ratio = number of small gold particles on the cytoplasmic surface/ number of large gold particles on the extracellular surface.

from a variety of species have a region corresponding to and having considerable homology with gamma 360-377 and the surrounding regions of the *Torpedo* receptor. Recent determination of the primary structure of the gamma-subunit from calf muscle has confirmed this prediction (44).

A major function of the cytoplasmic domains of the receptor subunits may be to interact with postsynaptic-specific, submembrane proteins. One such protein, the 43,000-mol-wt protein (28), has been shown by immunogold electron microscopy (34), and ultrastructural morphology (33) to be coextensively distributed with the receptor and to lie among or adjacent to the cytoplasmic regions of the receptor. The 43,000-mol-wt protein has also been chemically cross-linked to the beta subunit of the membrane-bound receptor (3). Thus, it may interact directly with cytoplasmic domains of the receptor, possibly serving to anchor the receptor at postsynaptic sites (4, 20, 30). The fact that mabs 264E and 274D react well with the receptor in nonextracted membranes (which retain the 43,000-mol-wt protein [34]) and in fixed electroplax (unpublished results) appears to rule out the possibility that association of the 43,000-mol-wt protein with the receptor could involve the region of gamma 360-377. In addition, mab 88B also reacts well with the postsynaptic membrane in fixed electroplax (unpublished results). Hence, there may be a substantial portion of the receptor in the region of gamma 360-377 that is not close to the 43,000-mol-wt protein. This conclusion probably cannot be extended to the homologous region of the delta-subunit on the basis of the present data, since mab 264E reacted less well with the nonextracted membranes than with the extracted membranes.

In previous experiments, several anti-AChR mabs, including 264E and 274D, reacted with the membrane-bound AChR only after alkaline extraction of purified receptor-rich membrane vesicles (13) and we suggested that the removal of peripheral membrane proteins uncovered the mab-binding sites. Although this point needs to be investigated for the other mabs that showed these binding characteristics, the results in this paper make it more likely that the important effect of alkaline extraction was to open the vesicles.

The polypeptide segment containing the cytoplasmic epitopes that we have identified has recently been proposed to contain sites of phosphorylation (40). According to this proposal, tyrosine-364 of the gamma subunit can be phosphorylated by a tyrosine kinase endogenous to the postsynaptic membrane preparation. In addition, phosphorylation on the gamma and delta subunits by a cAMP-dependent kinase and on the delta-subunit by protein kinase C may occur on serine residues close to but not contained within gamma 360-377 (40). Mabs 264E and 274D as well as others thought to bind to adjacent regions may be useful tools for verifying these sites of phosphorylation and for investigations of the effects of these modifications on receptor function.

We thank Karen Douville and Susan Klink for their technical assistance, Dr. Arthur Karlin for performing the sequence comparisons of the synthetic peptide and the receptor subunits, and Drs. Robert Stroud, Ellen Young and Jim Blake for providing us with the delta peptide 485-499.

This work was supported by National Institutes of Health grants (NS 14871 and NS 15293, to S. C. Froehner and R. Sealock, respectively), and a grant from the Muscular Dystrophy Association to S. C. Froehner.

Received for publication 24 July 1984, and in revised form 8 November 1984.

Note Added in Proof: Antibodies to synthetic peptides based on the region of the beta subunit that corresponds in position to gamma 340-349 and on the C-terminal region of the delta chain have recently been reported to bind to the cytoplasmic surface of the electrocyte postsynaptic membrane (E. F. Young, E. Ralston, J. Blake, J. Ramachandran, Z. W. Hall, and R. M. Stroud, 1985, *Proc. Natl. Acad. Sci. USA.* 82:In press).

REFERENCES

1. Anderson, D. J., S. J. Tzartos, W. Gullick, J. Lindstrom, and G. Blobel. 1983. Transmembrane orientation of an early biosynthetic form of acetylcholine receptor delta subunit determined by proteolytic dissection in conjunction with monoclonal antibodies. *J. Neurosci.* 3:1773–1784.
2. Atassi, M. Z. 1975. Antigenic structure of myoglobin. The complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins. *Immunochemistry.* 12:423–438.
3. Burden, S. J., R. L. DePalma, and G. S. Gottesman. 1983. Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta subunit and the 43kD subsynaptic protein. *Cell.* 35:687–692.
4. Cartaud, J., A. Sobel, A. Rousselet, P. F. Devaux, and J. P. Changeux. 1981. Consequences of alkaline treatment for the ultrastructure of the acetylcholine receptor-rich membranes from *Torpedo marmorata* electric organ. *J. Cell Biol.* 90:418–426.
5. Claudio, T., M. Ballivet, J. Patrick, and S. Heinemann. 1983. Nucleotide and deduced amino acid sequences of *Torpedo californica* acetylcholine receptor gamma subunit. *Proc. Natl. Acad. Sci. USA.* 80:1111–1115.
6. Conti-Tronconi, B. M., C. M. Gotti, M. W. Hunkapiller, and M. A. Raftery. 1982. Mammalian muscle acetylcholine receptor: a supramolecular structure formed by four related proteins. *Science (Wash. DC).* 218:1227–1229.
7. Devillers-Thiery, A., J. Giraudat, M. Bentaboulet, and J. P. Changeux. 1983. Complete mRNA sequence of the acetylcholine binding alpha-subunit from *Torpedo marmorata* acetylcholine receptor; a model for the transmembrane organization of the polypeptide chain. *Proc. Natl. Acad. Sci. USA.* 80:2067–2071.
8. Eldred, W. D., C. Zucker, H. J. Karten, and S. Yazulla. 1983. Comparison of fixation and penetration enhancement techniques for use in ultrastructural immunocytochemistry. *J. Histochem. Cytochem.* 31:285–292.
9. Finer-Moore, J., and R. M. Stroud. 1984. Amphipathic analysis and possible formation of the ion channel in an acetylcholine receptor. *Proc. Natl. Acad. Sci. USA.* 81:155–159.
10. Frens, G. 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. *Nature Phys. Sci.* 241:20–22.
11. Froehner, S. C. 1981. Identification of exposed and buried determinants of the membrane-bound acetylcholine receptor from *Torpedo californica. Biochemistry.* 20:4905–4915.
12. Froehner, S. C., and S. Rafto. 1979. Comparison of the subunits of *Torpedo californica* acetylcholine receptor by peptide mapping. *Biochemistry.* 18:302–307.
13. Froehner, S. C., K. Douville, S. Klink, and W. J. Culp. 1983. Monoclonal antibodies to cytoplasmic domains of the acetylcholine receptor. *J. Biol. Chem.* 258:7112–7120.
14. Froehner, S. C. 1984. Peripheral proteins of postsynaptic membranes from *Torpedo* electric organ identified with monoclonal antibodies. *J. Cell Biol.* 98:88–96.
15. Fujiwara, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.* 71:848–875.
16. Gentry, L. E., L. R. Rohrschneider, J. E. Casnellie, and E. G. Krebs. 1983. Antibodies to a defined region of $pp60^{src}$ neutralize the tyrosine-specific kinase activity. *J. Biol. Chem.* 258:11219–11228.
17. Guy, H. R. 1984. A structural model of the acetylcholine receptor channel based on partition energy and helix packing calculations. *Biophys. J.* 45:249–261.
18. Karlsson, E., H. Arnberg, and D. Eaker. 1971. Isolation of the principal neurotoxins of two *Naja naja* subspecies. *Eur. J. Biochem.* 21:1–16.
19. Kosower, E. M. 1983. Partial tertiary structure assignments for the beta, gamma, and delta subunits of the acetylcholine receptor on the basis of the hydrophobicity of amino acid sequences and channel location using single group rotation theory. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 155:245–247.
20. Lo, M. M. S., P. B. Garland, I. Lamprecht, and E. A. Barnard. 1980. Rotational mobility of the membrane-bound acetylcholine receptor of *Torpedo* electric organ measured by phosphorescence depolarization. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 111:407–410.
21. Merlie, J. P., R. Sebbane, S. Gardner, and J. Lindstrom. 1983. cDNA clone for the

alpha subunit of the acetylcholine receptor from the mouse muscle cell line BC_3H-1. *Proc. Natl. Acad. Sci. USA.* 80:3845–3849.

22. Muhlpfordt, H. 1982. The preparation of colloidal gold particles using tannic acid as an additional reducing agent. *Experientia (Basel).* 38:1127–1128.
23. Neubig, R. R., E. K. Krodel, N. D. Boyd, and J. B. Cohen. 1979. Acetylcholine and local anesthetic binding to *Torpedo* nicotinic postsynaptic membranes after removal of nonreceptor peptides. *Proc. Natl. Acad. Sci. USA.* 76:690–694.
24. Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, T. Kikyotani, T. Hirose, M. Asai, H. Takashima, S. Inayama, T. Miyata, and S. Numa. 1983. Primary structures of beta- and delta-subunit precursors of *Torpedo californica* acetylcholine receptor deduced from cDNA sequences. *Nature (Lond.).* 301:251–255.
25. Noda, M., H. Furutani, H. Takahashi, M. Toyosato, T. Tanabe, S. Shimizu, S. Kikyotani, T. Kayano, T. Hirose, S. Inayama, and S. Numa. 1983. Cloning and sequence analysis of calf cDNA and human genomic DNA encoding alpha-subunit precursor of muscle acetylcholine receptor. *Nature (Lond.).* 305:818–823.
26. Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, Y. Furutani, T. Hirose, H. Takashima, S. Inayama, T. Miyata, and S. Numa. 1983. Structural homology of *Torpedo californica* acetylcholine receptor subunits. *Nature (Lond.).* 302:528–532.
27. Popot, J. L., and J. P. Changeux. 1984. The nicotinic receptor of acetylcholine: structure of an oligomeric integral membrane protein. *Physiol. Rev.* 64:1162–1239.
28. Porter, S., and S. C. Froehner. 1983. Characterization and localization of the M_r 43,000 proteins associated with acetylcholine receptor-rich membranes. *J. Biol. Chem.* 258:10034–10040.
29. Raftery, M. A., M. W. Hunkapiller, C. D. Strader, and L. E. Hood. 1980. Acetylcholine receptor: complex of homologous subunits. *Science (Wash. DC).* 208:1454–1457.
30. Rousselet, A., J. Cartaud, and P. F. Devaux. 1981. Effects of temperature, lipid modification, and pH on the mobility of the major proteins of the receptor-rich membranes from *Torpedo marmorata. Biochem. Biophys. Acta.* 648:169–185.
31. Slot, J. W., and H. J. Geuze. 1981. Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* 90:533–536.
32. Sargent, P. B., B. E. Hedges, L. Tsavaler, L. Clemmons, S. Tzartos, and J. Lindstrom. 1984. Structure and transmembrane nature of the acetylcholine receptor in amphibian skeletal muscle as revealed by cross-reacting monoclonal antibodies. *J. Cell Biol.* 98:609–618.
33. Sealock, R. 1982. Cytoplasmic surface structure in postsynaptic membranes from electric tissue visualized by tannic acid mediated negative contrasting. *J. Cell Biol.* 92:514–522.
34. Sealock, R., B. E. Wray, and S. C. Froehner. 1984. Ultrastructural localization of the M_r 43,000 protein and the acetylcholine receptor in *Torpedo* postsynaptic membranes using monoclonal antibodies. *J. Cell Biol.* 98:2239–2244.
35. Sobel, A., M. Weber, and J. P. Changeux. 1977. Large-scale purification of the acetylcholine receptor protein in its membrane-bound and detergent extracted forms from *Torpedo marmorata* electric organ. *Eur. J. Biochem.* 80:215–244.
36. Sumikawa, K., M. Houghton, J. G. Smith, L. Bell, B. M. Richards, and E. A. Barnard. 1982. The molecular cloning and characterization of cDNA coding for the alpha-subunit of the acetylcholine receptor. *Nucleic Acids Res.* 10:5803–5822.
37. Tzartos, S., and J. M. Lindstrom. 1980. Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits. *Proc. Natl. Acad. Sci. USA.* 77:755–759.
38. Weber, K., P. C. Rathke, and M. Osborn. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and immunofluorescence. *Proc. Natl. Acad. Sci. USA.* 75:1820–1824.
39. Wray, B. E., and R. Sealock. 1984. Ultrastructural immunocytochemistry of particulate fractions using polyvinyl chloride microculture wells. *J. Histochem. Cytochem.* 31:1117–1120.
40. Huganir, R. L., K. Miles, and P. Greengard. 1984. Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. *Proc. Natl. Acad. Sci. USA.* 81:6968–6972.
41. Kao, P. N., A. J. Dwork, R.-R. J. Kaldany, M. L. Silver, J. Wideman, S. Stein, and A. Karlin. 1984. Identification of the alpha subunit half-cystine specifically labeled by an affinity reagent for the acetylcholine receptor binding site. *J. Biol. Chem.* 259:11662–11665.
42. Lindstrom, J., M. Criado, S. Hochschwender, J. L. Fox, and V. Sarin. 1984. Immunochemical tests of acetylcholine receptor subunit models. *Nature (Lond.).* 311:573–575.
43. Ratnam, M., and J. Lindstrom. 1984. Structural features of the nicotinic acetylcholine receptor revealed by antibodies to synthetic peptides. *Biochem. Biophys. Res. Commun.* 122:1225–1233.
44. Takai, T., M. Noda, Y. Furutani, H. Takahashi, M. Notake, S. Shimizu, T. Kayano, T. Tanabe, K. Tanaka, T. Hirose, S. Inayama, and S. Numa. 1984. Primary structure of gamma subunit precursor of calf muscle acetylcholine receptor deduced from the cDNA sequence. *Eur. J. Biochem.* 143:109–115.