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

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ABSTRACT Two monoclonal antibodies (mabs) previously prepared against Torpedo acetylcholine receptor are shown to recognize a synthetic nonadecapeptide corresponding to lys360-glut377 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 preceding 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.
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 M₄ 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 (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% NaN₃ 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 0-4°C. The peptide (4.5 μmol in 250 μl of 10 mM sodium phosphate, pH 7.4) was alkylated with 250 nmol of N-ethylmaleimide for 10 min and then treated with 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 BSAs. After incubation for 4 h, the conjugate was separated from the free peptide on a Sephadex G-50 column (dissolved in 200 μl 10 mM sodium phosphate, pH 7.4). The reaction was stopped by the addition of 5 μmol of ethanethiol and the iodinated protein was separated from sodium iodide by chromatography on a BioGel P60 column equilibrated with PBS. Specific radioactivities of 26 μCi/μmol for the conjugate and 20 μCi/μmol for control BSAs were obtained.

**Enzyme-Linked Immunosorbent 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/NaPO₄ containing 4% BSA for 30 min. and the plates were then washed with PBS/NaPO₄ containing 0.05% Tween. All subsequent washes were done with the same solution. The wells were then incubated with 50 μl of IgG diluted in PBS/NaPO₄ containing 1% BSA for 2 h, washed, and then incubated with antitoxin immunoglobulin conjugated to β-galactosidase (BRL Scientific, Gaithersburg, MD) (1:100) for 3 h. The wells were washed again and incubated with paranitrophenyl-β-galactoside (1 mg/ml) dissolved in 50 mM sodium phosphate buffer, pH 7.2, 1.5 mM MgCl₂, 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 88A147A; 20 nM for 264E; 139A, 240A, 245A, 262C, and 280B; 40 nM for 274D.

**IgM Immunoprecipitation of Radioiodinated Peptide-BSA Conjugate:** Mab IgG (10 μl) was incubated with 10 μl of 125I-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 bacteria (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 homogenate 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 (Sonicator Instrument Corp., Copiague, NY), pelleted, resuspended in water to their initial volume, and stored frozen in 50-μl aliquots. (b) Nonextraction membranes. Tissue was homogenized with 250-μl 25% sucrose 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 tubules. 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 530 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 antitoxin 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 coverslips 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 NaBH₄ (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 antitoxin 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 immunochromic 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. With large aggregations of membranes, fragments were often labeled by antireceptor mab but not by the α-toxin, certainly because of hindrance of access of reagents to the outer surfaces of the fragments (see Fig. 6). 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 identified.
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).

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.
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 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. S). The seven other anti-AChR mabs and mabs MPC-11 and MOPC-21 failed to immunoprecipitate iodinated conjugate. Since radiiodination 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).
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 ACh into rabbits were...
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...
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 coexclusively 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 electrophot (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 electrophot (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 delta-subunit on the basis of the homologous region of the delta-subunit.

The signal for bound antireceptor mabs is expressed relative to that for bound alpha-tubulin; ratio = number of small gold particles on the cytoplasmic surface per number of large gold particles on the extracellular surface.

Table 1

| Binding of Antireceptor mAbs to Postsynaptic Membranes | Ratio (+ peptide) | Ratio (− peptide) |
|--------------------------------------------------------|------------------|-----------------|
| Extracted membranes                                    |                  |                 |
| 264E                                                   | 1.69             | 0.43            |
| 264E + peptide (100 μM)                                | 0.72             | 0.43            |
| 274D                                                   | 0.56             | 0.18            |
| 274D + peptide (1 μM)                                  | 0.10             | 0.18            |
| Nonextracted membranes                                 |                  |                 |
| 264E                                                   | 0.81             |                 |
| 274D                                                   | 1.09             |                 |

The signal for bound antireceptor mabs is expressed relative to that for bound alpha-tubulin; ratio = number of small gold particles on the cytoplasmic surface per number of large gold particles on the extracellular surface.

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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).

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