Topological mapping of acetylcholine receptor: Evidence for a model with five transmembrane segments and a cytoplasmic COOH-terminal peptide

(Ion channel structure/antipeptide antibodies/immuno-electron microscopy/amphipathic helices)

ELLEN F. YOUNG*, EVELYN RALSTON†, JAMES BLAKE*, J. RAMACHANDRAN*, ZACH W. HALL†, AND ROBERT M. STRoud*

*Department of Biochemistry and Biophysics and †Division of Neurobiology, Department of Physiology, School of Medicine, University of California, San Francisco, CA 94143

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ABSTRACT Antibodies were raised against two synthetic peptides whose sequences correspond respectively to the COOH-terminal end (residues 501–516) of the protein encoded by the gene for the δ chain and to a proposed cytoplasmic region (residues 350–358) of the β chain of the acetylcholine receptor from Torpedo californica. Binding of the COOH-terminal antibody to the acetylcholine receptor in intact, receptor-rich vesicles was tested by radiimmunoassay and by precipitation with immobilized protein A. In both cases, binding was detected only after treatment of the vesicles with detergent, suggesting that the segment of the receptor that is recognized by this antibody is on the cytoplasmic side of the membrane. Electron microscopy of tissue from Torpedo electric organ labeled with colloidal gold-conjugated second antibodies established that both anti-receptor antibodies bind to the cytoplasmic surface of the postsynaptic membrane. These experiments give ultrastructural evidence that the COOH-terminal segment of the δ chain as well as residues 350–358 of the β chain are on the cytoplasmic surface. They strongly support a model in which each of the receptor subunits crosses the membrane five times and in which one transmembrane segment of each chain contributes to the formation of a central ion channel.

The nicotinic acetylcholine receptor (AcChO\r) is located in the postsynaptic membrane of cholinergic nerve terminals at neuromuscular junctions. It forms a gated ion channel that permits passage of ~10^4 sodium ions per msec upon agonist binding (1–3). The receptor is a pentamer of four different subunits (α–δ) with stoichiometry α3βγδ (4–6). All of the subunits span the membrane and occupy quasi-equivalent positions around the ion channel as seen by electron microscopy (7). The apparent molecular weights of the mature subunits, based on NaDodSO4/PAGE, are 40,000 (α), 49,000 (β), 60,000 (γ), and 65,000 (δ) (1, 2); based on the recently published cDNA sequences of the subunits, however, they are 53,649 (α), 56,060 (β), 58,053 (γ), and 59,792 (δ) before glycosylation or possible proteolytic processing (8–13). The receptor is glycosylated approximately eight times (14), so that the estimated total molecular weight is 295,000 (15). The deduced amino acid sequences reveal striking homology between the different subunits (6, 13, 15). This homology has led to the idea that each of the subunits contributes a similar peptide component to the formation of a central ion channel (3, 15). The subject of this paper is the topology of the peptide chain within the membrane as it relates to the ion channel. Several models of peptide folding across the membrane, having either four, five, or six membrane crossings per subunit, have been suggested (10, 12, 13, 15–18). Because the NH2-terminus of δ is in the extracellular portion of the protein (19), models with four or six membrane crossings orient both NH2- and COOH termini on the extracellular side of the membrane. Models with five membrane crossings place the COOH terminus on the cytoplasmic side. To locate the COOH terminus, we generated polyclonal antibodies against a synthetic hexadecapeptide corresponding to the COOH terminus of the δ chain, since it has the longest hydrophilic sequence as predicted from the cDNA sequence. With these antibodies, we show (i) that the COOH terminal sequence deduced from the cDNA clone of the δ subunit is present in the mature δ subunit of the acetylcholine receptor and (ii) that this amino acid sequence lies on the cytoplasmic side of the membrane. We also raised antibodies to a synthetic peptide corresponding to residues 350–358 of the β chain to further map the topology of the receptor chains with respect to the membrane. This portion of the mature β chain also was localized to the cytoplasmic side of the membrane as previously suggested (10, 12, 13, 15, 16, 18, 20, 21). There must, therefore, be an even number of transmembrane segments between residue 358 and the COOH terminus. These results are consistent with our hypothesis of a second, possibly channel-forming segment that crosses the membrane in this region.

METHODS

Anti-δ COOH Terminus Antibody (Antibody δ-273). The peptide Ac-Pro-Phe-Glu-Gly-Asp-Pro-Phe-Asp-Tyr-Ser-Ser-Asp-His-Pro-Arg-Gly-SH, corresponding to the sequence Ac-[GlyS516]δ-subunit-(501–516) (GlyS, thiglycine) of the 517-amino acid δ subunit, was synthesized by the solid-phase method (22), starting with tert-butyloxycarbonylthiglycine resin (23, 24). The peptide product was purified by partition chromatography (25) on Sephadex G-50 and characterized by paper electrophoresis, high-performance partition chromatography, and amino acid analysis. The peptide was selectively coupled at its COOH terminus to bovine serum albumin (BSA) by reaction with silver nitrate/N-hydroxysuccinimide (23, 24) as previously described for the coupling of corticotropinlyghycine (26) to BSA. Amino acid analysis of the peptide-BSA conjugate showed that 8 mol of peptide were coupled per mol of BSA.

Three male New Zealand White rabbits were immunized intradermally with δ COOH-terminal peptide-BSA conjugate

Abbreviations: AcChO\r, acetylcholine receptor; BSA, bovine serum albumin; mAb, monoclonal antibody.

The amino acid sequence numbers used are those obtained after alignment of all four subunits (20).
in Freund's complete adjuvant (27). To characterize the anti-
sperm, a sample of the peptide was treated with silver nitrate to
give Ac-Gly-β-subunit-(501-516), which was then ra-
dioiodinated by the chloramine-T method (27). A sensitive
radioimmunoassay capable of detecting 5-10 pg of peptide has
been established by using standard procedures (27). The anti-
sperm was used at a final dilution of 1:20,000. Peptide
bound to antibody was separated from free peptide by char-
coal adsorption (28). All three rabbits immunized showed
production of antibodies against the peptide. None of the an-
nimals showed symptoms of myasthenia gravis.

**Antibody Purification.** IgG-rich fractions of the collected
rabbit sera were obtained by precipitating the whole sera
with ammonium sulfate at 30% saturation. The resulting pre-
cipitate was washed with a 35%-saturated ammonium sulfate
solution and then dissolved in 20 mM phosphate buffer, pH
7.4. Buffer was adjusted to 0.15 M NaCl/20 mM phosphate,
pH 7.4 (P1/NaCl) and protein was concentrated by using
Amicon Centriflo membrane cones (nominal Mr retention
>25,000). An affinity column was prepared by coupling the
peptide to aminohexyl-Sepharose as previously described for
the preparation of β-endorphin-Sepharose (29). Whole
serum that was applied to the peptide-Sepharose column at
room temperature and washed with 10 column volumes of P1/
NaCl. The retained fraction was eluted with 100 mM gly-
cine-HCl, pH 3.2, containing BSA at 1 mg/ml. Eluate frac-
tions were neutralized immediately with a saturated
Na2HPO4 solution and were concentrated by using the Cen-
triflo cones.

**Anti-β Antibody (Antibody β-350).** The peptide Thr-Pro-
Ser-Pro-Asp-Ser-Lys-Pro-Thr-Cys was synthesized by the
Sequemat (Boston, MA). The first nine residues reproduce the
sequence 350-358 of the β subunit of the AcChoR. The pep-
tide was coupled to BSA with dianzotized benzidine (30) and
injected intradermally into a female New Zealand White rab-
bit. The serum was tested as described for the anti-β antibo-
dies. Dilutions of 1:100 and 1:1000 were used. There were no
signs of myasthenia gravis in the animal.

The antibody was affinity-purified on a column of peptide-
linked CN-Sepharose (Pharmacia) and eluted with 3 M sodi-
um thiocyanate. After extensive dialysis against P1/NaCl,
the antibodies were concentrated with Aquacide III (Callo-
chem).

**Control Antibodies.** Several antibodies were used through-
out this work as controls: monoclonal antibodies (mAbs)
210, 111, and 35 (referred to as L-210, L-111, and L-35), gen-
erated in rats (31, 32), were generously provided by J. Lind-
strom (Salk Institute, La Jolla, CA). mAb 88 (referred to as
F-88) which was generated in mice (33) and a rabbit polyclo-
nal anti-Torpedo californica AcChoR serum were gener-
ously provided by S. Froehner (Dartmouth Medical School,
Hanover, NH).

**Immunoblots.** Affinity-purified T. californica AcChoR or
a 1% (wt/vol) Triton X-100 extract of tissue that had been
interrupted and washed exactly as for electron microscopy was
electrophoresed on a NaDodSO4/12% polyacrylamide
gel. The proteins were electroeluted and transferred to a
nitrocellulose filter (34). The nitrocellulose filter was cut into
strips and each strip was incubated with first antibody fol-
lowed by 125I-labeled second antibody. Bands were detected
by autoradiography at ~70°C on Kodak X-Omat film.

**Antibody Binding Assay.** In vitro antibody-binding studies
were done using extracellular-side-out AcChoR vesicles (35,
36) in which disulfide bonds had been reduced and carba-
methylated with 2-mercaptoethanol and iodoacetamide to
ensure that all AcChoR was monomeric (37). Vesicle sam-
ple were divided into three aliquots: (i) intact control vesi-
cles, (ii) vesicles permeabilized with 1% saponin but other-
wise intact, and (iii) vesicles solubilized in 1% (wt/vol) Tri-
ton X-100 or 2% (wt/vol) sodium cholate. AcChoR solutions
were then diluted 40-fold with 20 mM phosphate, pH 7.2.
AcChoR, either in vesicles or solubilized (~200 fmol per as-
say), was incubated at room temperature for 3 hr with a
1:200 dilution of antibody (8, 273, β-350, F-88, or L-35) in
a final volume of 20 µl. All antibodies showed maximal reac-
tion at this concentration. AcChoR was labeled by addition
of 10 µl of 59 mM 125I-labeled α-bungarotoxin for 1 hr at room
temperature. After labeling, saponin-permeabilized vesicles were disrupted by adding 50 µl of 1% Triton X-100 in
P1/NaCl and incubating for 1 hr at room temperature.
P1/NaCl was added to vesicles to be kept intact or previously
disrupted. Protein A on lysed Staphylococcus aureus cells
(Pansorbin, Calbiochem; 100 µl of suspension) was used to
precipitate antibody-bound 125I-labeled toxin–receptor com-
plexes. After 30 min at room temperature, the precipitate
was sedimented through 1 ml of 1 M sucrose and 125I in
the pellets was determined in a Beckman γ counter. This assay
depends on accessibility of the immobilized protein A to the
Igs. Igs that bind to the portion of AcChoR on the exter-
nal surface of the vesicles are accessible to protein A-S. au-
reus, whereas Igs that bind internally are inaccessible un-
less the vesicles are solubilized with detergent. Thus, even
vesicles that are somewhat permeable to macromolecules
(38, 39) give the same results as sealed vesicles in this assay.

**Electron Microscopy and Immunofluorescence.** Membrane
sheets from T. californica electropores were prepared by slic-
ing fresh electric organ and further disrupting it by one
stroke in a Dounce homogenizer (40). The tissue was washed
three times in tissue buffer (280 mM NaCl/3.0 mM KCl/1.8
mM MgCl2/300 mM urea/100 mM sucrose/5.5 mM glu-
cose/40 mM Hepes/1% BSA, pH 7.2) by sedimenting in a
clinical centrifuge and then gently resuspending. All proc-
dures were executed at 4°C. Aliquots of the tissue were incu-
inated either with affinity-purified 8, 273 or β-350 or with con-
trol mAb L-210 or L-111 at a dilution of 1:2 for 12 hr and then
washed four times in tissue buffer. Both fluorescein-conju-
gated goat anti-rabbit (or anti-rat) IgG and colloidal gold-
conjugated goat anti-rabbit (or anti-rat) IgG were used as
second antibodies. For immunofluorescence, tissue was in-
cluded for 1 hr with the second antibody and rhodamine-
conjugated α-bungarotoxin, washed four times in tissue buff-
er, and then mounted on a slide with a cover slip for fluo-
rescence microscopy. For colloidal gold labeling, samples were
incubated overnight with 5 nm colloidal gold conjugated ei-
ther to goat anti-rabbit IgG or to goat anti-rat IgG (Janssen
Pharmaceutica, Beerse, Belgium) and then washed four
times in tissue buffer. L-35 was fixed in 4% paraformalde-
hyde/100 mM sodium cacodylate for 1 hr on ice and then
washed three times in 100 mM sodium cacodylate. Sam-
ple were post-stained with 2% (wt/vol) osmium tetrox-
ide/100 mM sodium cacodylate at room temperature, dehy-
drated in ethanol, and embedded in Epon (60°C). Silver
gray sections were adsorbed to cleaned copper grids, post-
stained with 1% (wt/vol) uranyl acetate, and coated with car
Carbon. Electron microscopy was carried out using an 80 kV ac-
celerating voltage on a Phillips EM-400 microscope.

**RESULTS AND DISCUSSION**

Immunoblots with either purified AcChoR or a crude mem-
brane extract of Torpedo electric organ showed that affinity-
purified antibodies 8, 273 and β-350 bind to the β and δ
chains, respectively, of the mature AcChoR. There was es-
entially no cross-reactivity to the other chains of AcChoR or
other proteins (Fig. 1). The four subunits were checked for
homology with the synthetic peptides at regions other than the
COOH terminus or the cytoplasmic region near residue 350: no exact match of more
than three consecutive amino acids was found. The greatest
similarity of sequences occurred between the δ chain peptide
and the homologous COOH terminus of the ϵ chain, though
To determine directly the sidedness of binding to the AcChoR in outside-out vesicles, we used an immunoprecipitation assay that depends on accessibility of bound antibody to immobilized protein A. Two antibodies were used as controls: mAb L-35, which binds to the main immunogenic region on the α chain of the synaptic side of the membrane; and mAb F-88, which binds both γ and δ on the cytoplasmic side. mAb L-35 gave 100% immunoprecipitation of the 125I-labeled receptors when incubated with either intact or detergent-treated preparations. In contrast, mAb F-88 precipitated <10% of labeled AcChoR from intact vesicles, presumably due to AcChoR in membrane sheets rather than in vesicles. More than 55% were precipitated from detergent-solubilized vesicles (Fig. 3). The failure to achieve 100% precipitation with mAb F-88 is unexplained; higher antibody concentrations did not increase the amount of AcChoR precipitated, and the results were the same whether vesicles were solubilized before or after application of the first antibody.

The pattern obtained with anti-δ COOH-terminus antibodies (δ-273) was essentially identical to that obtained with mAb F-88: precipitation of AcChoR from intact vesicles increased from 8% to 45% upon solubilization with cholate. Similar results were obtained using β-350, with precipitation of up to 26% of solubilized AcChoR. When intact but leaky vesicles were substituted for saponin-treated vesicles, up to ~50% of AcChoR could be precipitated by δ-273 or by F-88. These results indicate that both δ-273 and β-350 recognize an internal (cytoplasmic) site on the AcChoR.

To localize the sites of binding of the antibodies to the AcChoR in tissue rather than vesicles and without recourse to detergent treatment, we examined sections of Torpedo electric organ by immuno-electron microscopy. Disrupted tissue was incubated with anti-peptide antibody followed by second antibody conjugated to colloidal gold. Basal lamina and portions of presynaptic membrane with associated synaptic vesicles were seen to adhere to the postsynaptic membrane (Fig. 4) allowing its two sides to be unambiguously identified. Both antibodies δ-273 and β-350 bound exclusively to the cytoplasmic side (Figs. 4 and 5). The results obtained with the two antibodies, although similar, showed differences in detail. β-350 showed a relatively uniform distri-

Fig. 1. Immunoblots. Antibodies δ-273 (lanes a and d), β-350 (lanes b and e) and a rabbit anti-Torpedo AcChoR serum (lanes c and f) were incubated with nitrocellulose blots of electrophoresed affinity-purified AcChoR (lanes a–c) or a Triton X-100 extract of electric organ prepared as for electron microscopy (lanes d–f), followed by incubation with 125I-labeled-goat anti-rabbit IgG and autoradiography. No cross-reactivity was seen on immunoblots. Therefore, the antibodies probably bind only to sections of the mature polypeptide chains that correspond to the DNA sequence on which the peptides were based; in the case of δ-273, this corresponds to the COOH terminus.

Binding of δ-273 to Torpedo AcChoR was measured using a radioimmunoassay with the 125I-labeled peptide. From saturation analysis of the binding of the iodinated peptide to the anti-δ COOH-terminus antibody, an apparent association constant of 3.3 × 10⁹ M⁻¹ was obtained. The unlabeled peptide caused 40% displacement of the labeled ligand at a concentration of 0.25 nM (Fig. 2). Sealed AcChoR vesicles failed to compete with the labeled peptide even at a concentration of 25 nM. On the other hand, vesicles made permeable by treatment with saponin competed very effectively, causing 40% displacement of 125I-labeled peptide at a concentration of 10 nM. Reduced and carbamoylmethylated (monomeric) AcChoR was used in all in vitro binding assays; in immunoprecipitation, δ-273 showed a strong preference for monomeric over dimeric AcChoR (unpublished data).

Fig. 2. Competition between the synthetic δ C-terminal peptide and AcChoR. 125I-labeled Ac( Gly)₃–δ-subunit-(501–516) was incubated with the antibody δ-273 at a 1:20,000 dilution for 24 h at 20°C followed by competition with AcChoR in sealed intact vesicles (○), saponin-permeabilized AcChoR vesicles (△), and non-iodinated peptide (●).

Fig. 3. In vitro binding assays. Intact AcChoR vesicles (V), saponin-permeabilized AcChoR vesicles (S), and AcChoR vesicles solubilized in 2% sodium cholate (C) were incubated with antibody δ-273, F-88, or L-35, each at 1:200 dilution as explained in Methods. AcChoR was labeled with 125I-labeled α-bungarotoxin and AcChoR–antibody complexes were precipitated by adsorption onto protein A–S. aureus particles.
Fig. 4. Electron micrographs showing binding of δ-273, anti-δ COOH-terminus antibody (A); L-210, a mAb that binds on the extracellular portion of the AcChoR (B); antibody β-350, which binds to residues 350–358 of AcChoR β subunit (C); and L-111, a mAb that binds to the cytoplasmic side of the T. californica postsynaptic membrane. Antibodies were visualized with 5 nm colloidal gold (seen as black beads) conjugated to goat anti-rabbit IgG (or anti-rat IgG). Gold beads can be seen clearly on the cytoplasmic (c) side of the postsynaptic membrane (indicated by arrow) in the case of antibodies δ-273, β-350, and L-111, whereas antibody L-210 labels the synaptic side of the postsynaptic membrane. Even labeling of the postsynaptic membrane was observed with all of the AcChoR antibodies tested with the exception of δ-273, which appeared to label extensions of the postsynaptic membrane more heavily. The bar is equivalent to 2000 Å and applicable to all four micrographs. Sections were stained with uranyl acetate.

**CONCLUSION**

Our results show that the COOH terminus of the δ subunit of AcChoR and the sequence 350–358 of the β chain are located on the cytoplasmic side of the postsynaptic membrane, indicating that the polypeptide chain of AcChoR δ subunit crosses the lipid bilayer an odd number of times. The strong sequence homology between chains of AcChoR suggests a common topology for all of the subunits (13); thus, finding β-350 on the cytoplasmic side implies that there are an even number of membrane crossings between residue 358 and the COOH terminus in the common threading pattern (Fig. 6).

![Schematic drawing showing the predicted secondary structure for the receptor subunits and the “five-crossing” model of polypeptide chain threading through the bilayer (15).](image-url)
Within each subunit sequence there are only four obviously hydrophobic regions long enough to span the membrane (10, 12, 13). A fifth sequence, identified by amphipathic analysis, has also been postulated to span the membrane. This sequence can form an α-helix that is hydrophobic on one side and highly charged on the other (3, 15, 18). In conjunction, the homologous sequences from the five subunits, presented as amphipathic helices oriented so that the charged side of each helix faces the center, could form the ion channel across the most hydrophobic part of the plasma membrane.

Our results provide evidence that there must be a fifth membrane spanning in addition to the four hydrophobic spanning regions. Experiments that rely on detergent to enhance antibody labeling of AcChoR cannot be proof of a cytoplasmic epitope, because the possibility of loosening the target peptide at the exterior surface, also leading to enhancement, cannot be excluded. Our results are consistent with the idea that the ion channel in the AcChoR is formed by five homologous amphipathic helices, each of which contributes charged residues to the lining of a water-filled ion-conducting channel (Fig. 7). Model-building and energy calculations with the program AMBER (41) show that this structure can catalyze the passage of hydrated sodium ions or organic cations known to pass through the AcChoR channel (unpublished observations).

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