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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)

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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 radioimmunoassay 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 (AcChoR) is located in the postsynaptic membrane of cholinergic nerve terminals at neuromuscular junctions. It forms a gated ion channel that permits passage of $\approx 10^4$ sodium ions per msec upon agonist binding (1–3). The receptor is a pentamer of four different subunits (α–δ) with stoichiometry $\alpha_2\beta\gamma\delta$ (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 $NaDodSO_4$/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 NH_2 terminus of δ is in the extracellular portion of the protein (19), models with four or six membrane crossings orient both NH_2- 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-[$GlyS^{516}$]δ-subunit-(501–516) (GlyS, thioglycine) of the 517-amino acid δ subunit, was synthesized by the solid-phase method (22), starting with *tert*-butyloxycarbonylthioglycine 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 corticotropinylthioglycine (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

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Abbreviations: AcChoR, 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 antiserum, a sample of the peptide was treated with silver nitrate to give Ac-[Gly^{516}]δ-subunit-(501–516), which was then radioiodinated 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 antiserum was used at a final dilution of 1:20,000. Peptide bound to antibody was separated from free peptide by charcoal adsorption (28). All three rabbits immunized showed production of antibodies against the peptide. None of the animals 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 precipitate 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 (P_i/NaCl) and protein was concentrated by using Amicon Centriflo membrane cones (nominal M_r 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 was applied to the peptide-Sepharose column at room temperature and washed with 10 column volumes of P_i/NaCl. The retained fraction was eluted with 100 mM glycine·HCl, pH 3.2, containing BSA at 1 mg/ml. Eluate fractions were neutralized immediately with a saturated Na_2HPO_4 solution and were concentrated by using the Centriflo cones.

Anti-β Antibody (Antibody β-350). The peptide Thr-Pro-Ser-Pro-Asp-Ser-Lys-Pro-Thr-Cys was synthesized by Sequemat (Boston, MA). The first nine residues reproduce the sequence 350–358 of the β subunit of the AcChoR. The peptide was coupled to BSA with diazotized benzidine (30) and injected intradermally into a female New Zealand White rabbit. The serum was tested as described for the anti-δ antibodies. 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 sodium thiocyanate. After extensive dialysis against P_i/NaCl, the antibodies were concentrated with Aquacide III (Calbiochem).

Control Antibodies. Several antibodies were used throughout this work as controls: monoclonal antibodies (mAbs) 210, 111, and 35 (referred to as L-210, L-111, and L-35), generated in rats (31, 32), were generously provided by J. Lindstrom (Salk Institute, La Jolla, CA). mAb 88 (referred to as F-88) which was generated in mice (33) and a rabbit polyclonal anti-*Torpedo californica* AcChoR serum were generously 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 disrupted and washed exactly as for electron microscopy was electrophoresed on a $NaDodSO_4$/9% polyacrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose filter (34). The nitrocellulose filter was cut into strips and each strip was incubated with first antibody followed by ^{125}I-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 carbamoylmethylated with 2-mercaptoethanol and iodoacetamide to ensure that all AcChoR was monomeric (37). Vesicle samples were divided into three aliquots: (*i*) intact control vesicles, (*ii*) vesicles permeabilized with 1% saponin but otherwise intact, and (*iii*) vesicles solubilized in 1% (wt/vol) Triton 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 assay), was incubated at room temperature for 3 hr with a 1:200 dilution of antibody (δ-273, β-350, F-88, or L-35) in a final volume of 20 μl. All antibodies showed maximal reaction at this concentration. AcChoR was labeled by addition of 10 μl of 59 mM ^{125}I-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 P_i/NaCl and incubating for 1 hr at room temperature. P_i/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 ^{125}I-labeled toxin–receptor complexes. After 30 min at room temperature, the precipitate was sedimented through 1 ml of 1 M sucrose and ^{125}I in the pellets was determined in a Beckman γ counter. This assay depends on accessibility of the immobilized protein A to the IgGs. IgGs that bind to the portion of AcChoR on the external surface of the vesicles are accessible to protein A-*S. aureus*, whereas IgGs that bind internally are inaccessible unless 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* electroplax were prepared by slicing 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 $MgCl_2$/300 mM urea/100 mM sucrose/5.5 mM glucose/40 mM Hepes/1% BSA, pH 7.2) by sedimenting in a clinical centrifuge and then gently resuspending. All procedures were executed at 4°C. Aliquots of the tissue were incubated either with affinity-purified δ-273 or β-350 or with control 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-conjugated 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 incubated for 1 hr with the second antibody and rhodamine-conjugated α-bungarotoxin, washed four times in tissue buffer, and then mounted on a slide with a cover slip for fluorescence microscopy. For colloidal gold labeling, samples were incubated overnight with 5 nm colloidal gold conjugated either to goat anti-rabbit IgG or to goat anti-rat IgG (Janssen Pharmaceutica, Beerse, Belgium) and then washed four times in tissue buffer. Tissue was fixed in 2% (vol/vol) glutaraldehyde/100 mM sodium cacodylate for 1.5 hr on ice and then washed three times in 100 mM sodium cacodylate. Samples were post-stained with 2% (wt/vol) osmium tetroxide/100 mM sodium cacodylate at room temperature, dehydrated 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 carbon. Electron microscopy was carried out using an 80 kV accelerating voltage on a Phillips EM-400 microscope.

RESULTS AND DISCUSSION

Immunoblots with either purified AcChoR or a crude membrane extract of *Torpedo* electric organ showed that affinity-purified antibodies δ-273 and β-350 bind to the δ and β chains, respectively, of the mature AcChoR. There was essentially no cross-reactivity to the other chains of AcChoR or to other proteins (Fig. 1). The amino acid sequences of all 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 γ chain, though

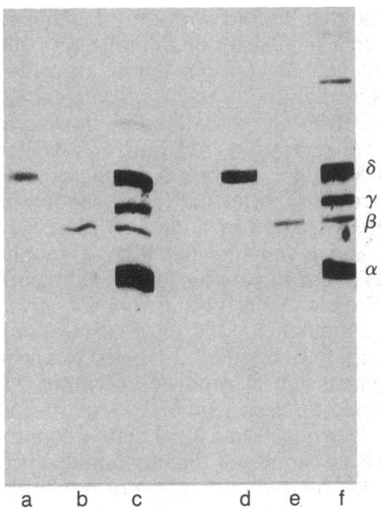

FIG. 1. Immunoblots. Antibodies δ-273 (lanes a and d), β-350 (lanes b and e) and a rabbit anti-*Torpedo* AcChoR serum (lanes c and f) were incubated with nitrocellulose blots of electrophoresed affinity-purified AcChoR (lanes a–c) or a Triton X-100 extract of electric organ prepared as for electron microscopy (lanes d–f), followed by incubation with ^{125}I-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 ^{125}I-labeled peptide. From saturation analysis of the binding of the iodinated peptide to the anti-δ COOH-terminus antibody, an apparent association constant of 3.3×10^9 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 ^{125}I-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).

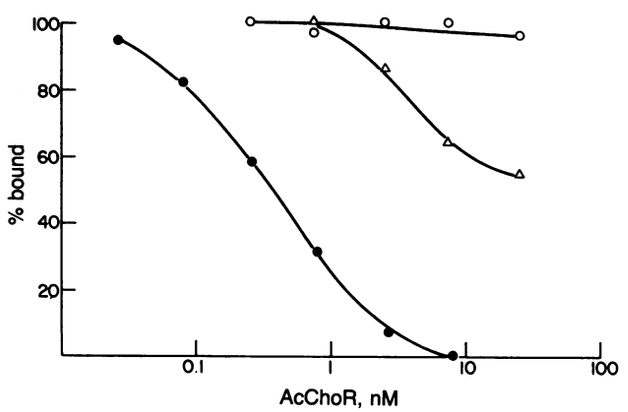

FIG. 2. Competition between the synthetic δ C-terminal peptide and AcChoR. ^{125}I-labeled Ac[Gly^{516}]δ-subunit-(501–516) was incubated with the antibody δ-273 at a 1:20,000 dilution for 24 hr at 20°C followed by competition with AcChoR in sealed intact vesicles (○), saponin-permeabilized AcChoR vesicles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

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 on 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 ^{125}I-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-

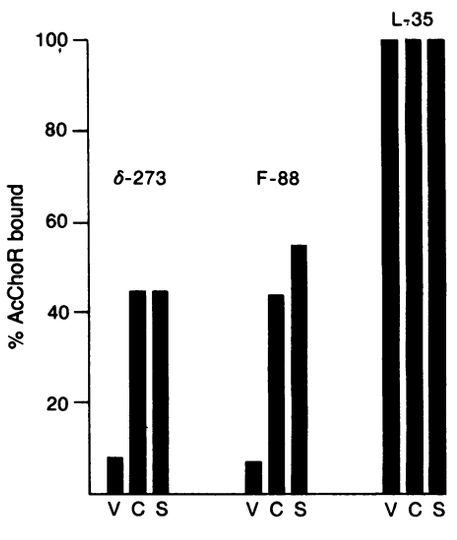

FIG. 3. *In vitro* binding assays. Intact AcChoR vesicles (V), saponin-permeabilized AcChoR vesicles (S), and AcChoR vesicles solubilized in 2% sodium cholate (C) were incubated with antibody δ-273, F-88, or L-35, each at 1:200 dilution as explained in *Methods*. AcChoR was labeled with ^{125}I-labeled α-bungarotoxin and AcChoR–antibody complexes were precipitated by adsorption onto protein A-*S. aureus* particles.

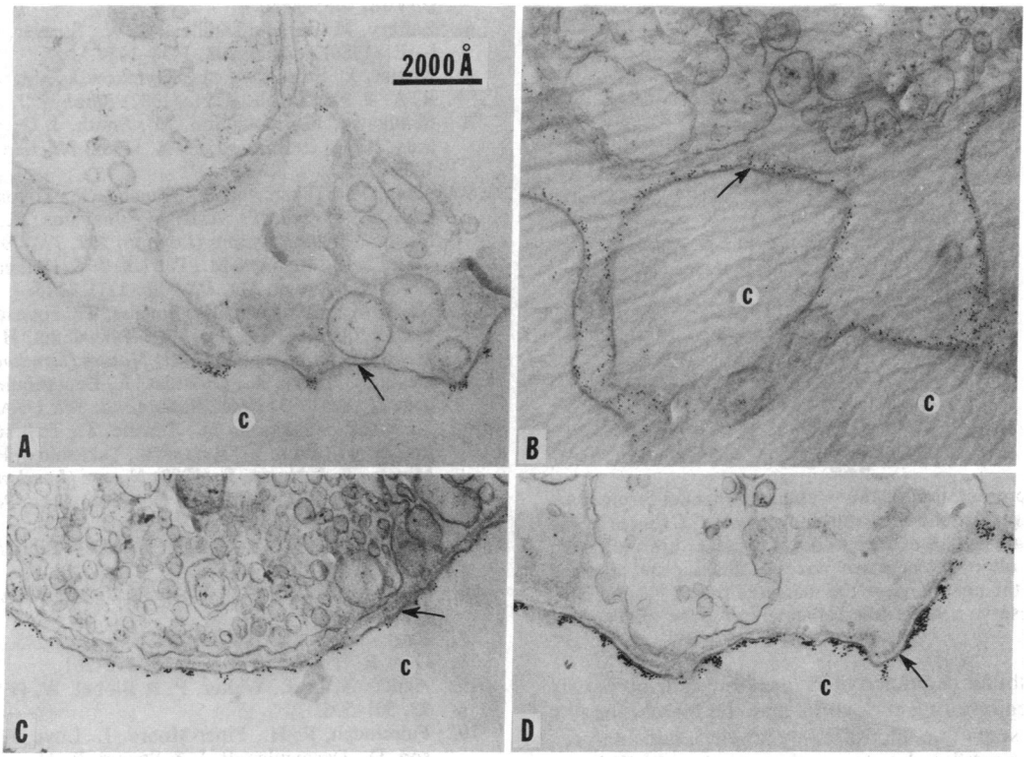

FIG. 4. Electron micrographs showing binding of δ-273, anti-δ COOH-terminus antibody (*A*); L-210, a mAb that binds on the extracellular portion of the AcChoR (*B*); antibody β-350, which binds to residues 350–358 of AcChoR β subunit (*C*); and L-111, a mAb that binds to the cytoplasmic side of the *T. 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.

bution of label like that seen with mAb L-111 (Fig. 4 *C* and *D*), which binds to the cytoplasmic surface (32). δ-273 showed a more uneven distribution, suggesting that in some regions the antigenic site may be physically or conformationally less accessible. Both sides of the membrane were accessible to anti-receptor antibodies as well as to gold-conjugated second antibodies, since labeling of the synaptic side was obtained with mAb L-210, which binds to an extracellular site on the AcChoR (Fig. 4*B*). Negligible labeling was seen in the absence of first antibody and, in all cases, membranes other than the postsynaptic membrane showed essentially no labeling. The specificity of binding of the antibodies to the postsynaptic membrane was confirmed by immunofluorescence (not shown). Both antibodies δ-273 and β-350 showed a staining pattern, revealed by fluorescein-conjugated second antibody, that was identical to that seen with rhodamine-conjugated α-bungarotoxin.

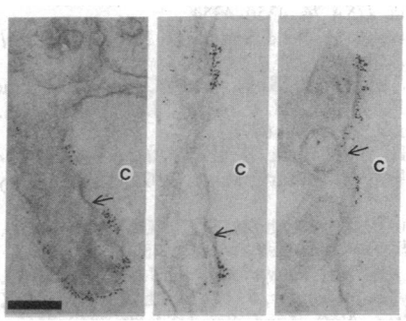

FIG. 5. Electron micrographs showing binding of δ-273 to the cytoplasmic (c) side of the postsynaptic membrane (indicated by arrow). The stain is uranyl acetate. (Bar = 1000 Å.)

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).

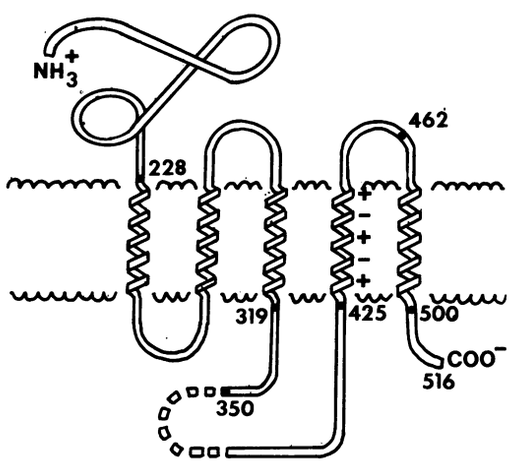

FIG. 6. Schematic drawing showing the predicted secondary structure for the receptor subunits and the "five-crossing" model of polypeptide chain threading through the bilayer (15).

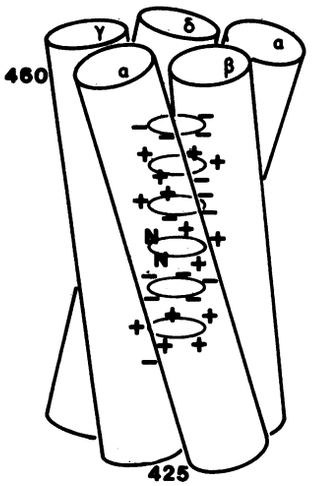

FIG. 7. A representation of the postulated channel-forming sequences as arranged around the central ion channel. Charges inside the transmembrane region of the proposed channel are indicated. Note that charge alternates between planes of positive and negative charge, and that the net charge in the indicated region is zero. This structure can preserve a water-filled channel inside the AcChoR rosette.

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 crossing 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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